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EFFECT OF pH AND ORGANIC COMPOUNDS ON NITROGEN FIXATION BY RED CLOVER

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INTRODUCTION

Symbiotic nitrogen fixation by leguminous plants depends on a number of different factors *viz* a) presence of cells of an effective Rhizobium strain in the culture medium, b) increase in numbers of Rhizobium cells in the rhizosphere, c) infection of the roots by the bacteria, d) growth of the nodules, e) activity of the nodules, and f) longevity of the nodules. Environmental conditions may affect nitrogen fixation by way of a more or less specific influence on one of the components. Boron deficiency, for instance, inhibits nitrogen fixation by preventing the growth of the nodule tissue. It does not seriously affect the mechanism of nitrogen fixation [5]. Molybdenum deficiency, however, primarily affects the nitrogen-fixing process, but it has only a slight effect on the development of the nodules [4].

As to the pH of the culture medium, it is a well-known fact that most leguminous plants grow less favourably in acid media than under neutral or slightly alkaline conditions [13]. This is mainly due to a reduced nitrogen fixation as may be concluded from the improved growth at low pH upon the addition of combined nitrogen. There exists much variation, however, amongst different species of leguminous plants as to the effect of pH on growth in the presence of combined nitrogen.

The purpose of the present study was to elucidate the observed beneficial effect of added calcium carbonate and of some organic compounds on symbiotic nitrogen fixation by red clover, growing on acid soil. The investigations started in 1957 when mixtures of grass and red clover were sown on a series of plots of varying pH.

The growth of the clover appeared to be luxurious at pH 6.6 but extremely poor at pH values of 5.0 and lower. The latter was due to the absence of root nodules.

On some acid plots which had been treated with stable manure a few years before starting the clover experiments, the red clover was well nodulated and an excellent growth was made, notwithstanding the pH of the soil was somewhat lower than that of untreated plots, where the clover suffered from severe nitrogen deficiency. This demonstrated that the effect of low pH on nodulation was eliminated by some unknown substance supplied by the stable manure.

To investigate the effect of pH and of various organic compounds on symbiotic nitrogen fixation in red clover a number of pot experiments were undertaken with sandy soils and with culture solutions.

METHODS

Field experiments

Red clover was grown on a number of plots with different pH values as a pure culture in 1959 and in mixture with perennial rye grass (*Lolium perenne*) in 1957 and 1958. These plots had been laid down in triplicate more than thirty years before on a poor sandy soil (organic matter 2%) in the garden of the Laboratory of Microbiology by applying calcium carbonate in the following amounts: 1250, 2500, 3750, 5500, and 11000 kg per ha. For maintaining the pH levels calcium carbonate was added periodically. The basic dressing consisted of superphosphate (100 kg P_2O_5 per ha) and potassium sulphate (125 kg K_2O per ha).

No clover had been grown on the plots in the years preceding the experiments and no effort was made to introduce Rhizobium during the 1957–'59 experiments or earlier.

Pot experiments

Soil cultures. Use was made of Neubauer glass jars containing 0.5 kg of sandy soil. The basic dressing for each pot consisted of 300 mg KH_2PO_4, 20 mg Na_2HPO_4 and 125 mg $MgSO_4.7H_2O$. Approximately twenty plants of red clover were grown per pot. In order to prevent the introduction of foreign *Rhizobium trifolii* cells into the soil employed, the glass containers, watering tubes, and nutrients were sterilized. The seeds were desinfected by treatment during 15 minutes with a 3 per cent H_2O_2 solution containing a few drops of the detergent "Teepol" per 100 ml of solution. Subsequently they were washed 5 times with sterile water and transferred to the soil. The pots were wrapped in cellophane paper until the seedlings had reached a

height of about two cm. Then the cellophane was removed and the soil was covered with a one-cm layer of glass sand, which had been paraffined by treating 10 kg sand with 10 g of paraffin wax dissolved in one liter benzene. After evaporation of the benzene this sand had been sterilized for several hours at 100° C. For watering the plants, sterile distilled water was added via a glass tube filled with sterile, coarse sand and sealed with a cotton plug. During autumn and winter the plants were kept in a greenhouse where they were provided with artificial light from 4 a.m. to sunrise and from sunset until 22 p.m. During spring and summer they were kept outdoors during day time and transferred to the greenhouse overnight and in rainy weather.

Cultures in nutrient solution. The culture solution employed had the following composition: KH_2PO_4 80 mg, K_2HPO_4 200 mg, $Na_2HPO_4.2H_2O$ 50 mg, $MgSO_4.7H_2O$ 250 mg, $CaSO_4.2H_2O$ 250 mg, NH_4NO_3 30 mg, $FeCl_3.6H_2O$ 25 mg, $MnSO_4.1H_2O$ 1 mg, $ZnSO_4.7H_2O$ 0.25 mg, $CuSO_4.5H_2O$ 0.25 mg, H_3BO_3 0.25 mg, and $Na_2MoO_4.2H_2O$ 0.25 mg per litre of distilled water.

Counting of Rhizobium trifolii in soil

To estimate the number of Rhizobium cells in soil, 10-g samples were suspended in 100 ml of a sterile solution of the following composition: K_2HPO_4 1 g, $Ca(H_2PO_4)_2$ 0.25 g, $(NH_4)_2SO_4$ 0.25 g and $MgSO_4.7H_2O$ 0.25 g per litre of distilled water. The suspension was shaken vigorously for 5 minutes before being diluted by serial transfer of 1 ml suspension to 9 ml of fresh, sterile medium until a dilution was attained of 10^9. Each dilution was made in duplicate. One-ml portions of the various dilutions were transferred to sterile 20-days old red-clover plants growing on agar slopes in 16 × 155 mm tubes. Each tube contained 7 ml of the nutrient solution, employed in the water cultures, solidified with 0.6 per cent Davis agar.

The plants were incubated during 45 days in the light in a greenhouse before being examined for effective nodules. When no nodules had formed, the plants were small and had an almost yellow leaf colour. Nodulated plants always had a dark-green colour indicating the presence of an effective Rhizobium strain. When effective nodules were formed at a certain dilution, it was assumed that at least one cell of an effective strain had been introduced (cf Hely *et al.*[2] and *Purchase* and *Nutman*[7]). The number of virulent bacteria was calculated according to the method described in "Standard methods of water analysis" [9].

Of each treatment two pots were sampled. In the case of unplanted soil the samples at each sampling time were taken from the same pots, the soil of which was mixed thoroughly. Of the planted pots two were harvested and sampled at each counting date (two samples per pot). To include the rhizosphere soil in the sample, the roots were transferred onto a coarse sieve where the bulk of the adhering soil was removed carefully in such a way that the roots and the nodules were left on the sieve. Thereafter the roots were washed twice with 20 ml sterile water and the washings added to the soil. After thorough mixing the samples were taken.

Preparation of extracts of Rhizobium, yeast, and stable manure

Sterilized Rhizobium cells. Effective and ineffective strains of *Rhizobium trifolii* were cultivated on agar plates in petri dishes of 11 cm in diameter. The agar media had the following composition: K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, $CaCO_3$ 3 g, mannitol 1.5 g, glutamic acid 0.2 g, Difco yeast extract 1 g, cornsteep liquor 1 ml, soil extract 850 ml (prepared from 425 g clay soil), distilled water 150 ml, Davis agar 10 g. After three to four days' incubation at 30° C, the slimy bacteria layer of 16 plates was collected and suspended in 100 ml water. The suspensions were autoclaved twice at 110° C for 10 minutes. Their nitrogen content varied from 0.4 to 0.6 mg per ml.

Yeast extract. 100 g of bakers yeast was heated with 200 ml water for 10 minutes at 90° C. The suspension was centrifuged and the clear solution, containing approximately 1 mg N per ml, autoclaved at 110° C for 10 minutes and used for the pot experiments.

Stable-manure extract. One kg fresh stable manure containing approximately 24 per cent dry matter was cut to small pieces and suspended in 3 l distilled water. The suspension was shaken mechanically for 18 h. Thereafter it was squeezed through cheese cloth and filtered on a Buchner funnel through asbestos. The filtrate was concentrated *in vacuo* to 150 ml. The latter solution contained 0.75 mg N per ml.

RESULTS

1. *Effect of added calcium carbonate on growth and nitrogen fixation of red clover on acid sandy soils*

a. Field experiments. The effect of pH on yield of red clover grown on plots treated with different amounts of calcium carbonate is shown in Table 1. It will be seen that an adequate growth of the clover took place at pH 6.4 and 6.8. At pH 4.7 and 5.0 the development of the plants was poor due to the absence of root nodules. That nitrogen deficiency was the main cause of the restricted growth of the clover on the acid plots may also be concluded from the results obtained with added fertilizer nitrogen. At pH values of 6.4 and higher normal nodulation occurred as a result of which the plants were adequately supplied with nitrogen. Since inoculation of the seed or the soil on these plots had not been performed and no clover crops had been grown there before, this demonstrates that Rhizobium cells were present in the neutral and alkaline soils but in the acid soils they were either absent or unable to initiate the formation of nodules.

To see which component of the symbiotic nitrogen-fixing system

was affected by a low pH, a number of pot experiments with acid soils were conducted.

b. Pot experiments. Acid sandy soil (pH 5.1, organic matter 2%), from one of the above-mentioned plots was mixed with 1 g sterilized calcium carbonate, 1 g per pot, and transferred to the glass containers. Some pots with acid soil and some with calcium

TABLE 1

Effect of pH and fertilizer nitrogen on yield (fresh weight, q/ha *) of uninoculated red clover (first cutting), grown under field-experiment conditions

pH of the soil	No nitrogen added		120 kg N/ha ‡
	1957 ***	1959 †	1959 †
4.7	—	65	193
5.0	4	29	178
5.0 **	242	—	—
5.1	7	70	189
5.6	27	85	182
6.4	200	158	238
6.8	303	169	263

* 1 q = 1 quintal = 100 kg.
** Treated with stable manure some years before starting the experiment.
*** Single values.
† Averages of duplicate values.
‡ In the form of ammonium-nitrate limestone.

carbonate-treated soil were inoculated with a suspension of an effective strain of *Rhizobium trifolii*, the others were left uninoculated. Some further pots with acid soil were provided with 42 mg nitrogen as ammonium nitrate. The soil of the latter pots was not inoculated with Rhizobium. Red clover was grown as described above. The experiment was started on 28 August and finished on 19 December 1957.

In the absence of added calcium carbonate or ammonium nitrate the growth of the uninoculated clover was very poor. The plants were small and yellow-green due to nitrogen deficiency; root nodules were absent. A few plants of these control pots showed a somewhat greener leaf colour and better development than the others. This was brought about by the presence of a few nodules which, however, did not give rise to a much better growth. Apparently the bacteria which had given rise to these few nodules had been unable to spread in the rhizosphere of the clover roots in the acid soil.

The plants on the uninoculated, calcium carbonate-treated soil at first showed a more or less similar appearance. After an initial growth depression owing to nitrogen deficiency some plants turned green and developed vigorously (Plate 1A). The improved growth, which was a result of the formation of nodules, gradually spread throughout the pot. In contrast to the acid soil the few Rhizobium cells which presumably were present originally, now were able to increase in numbers and to give rise to the formation of large numbers of nodules.

In the case of plants inoculated with a suspension of Rhizobium cells, formation of nodules and development of the clover plants on the acid soil were quite satisfactory. This demonstrates that the low pH apparently did not depress the infection of the roots and the growth and the activity of the nodules (Plate 1B).

Uninoculated plants treated with ammonium nitrate initially made much better growth than those of the control pots. The amount of added nitrogen was too low, however, to supply the plants with adequate amounts (Table 2). Inoculated plants dressed with

TABLE 2

Effect of added $CaCO_3$ and NH_4NO_3 on yield (g dry matter per pot) of inoculated and uninoculated red clover, grown on an acid sandy soil

Treatment	Uninoculated	Inoculated
First experiment *		
Control	0.5	3.0
1 g $CaCO_3$ per pot	1.7	2.8
NH_4NO_3	0.6	2.0
Second experiment **		
Control	0.2	—
1 g $CaCO_3$ per pot	11.3	—

* Single values.

** Averages of quadruplicate values.

Plate 1. A. Effect of added calcium carbonate on nitrogen fixation and growth of red clover on acid soil. *Left* no $CaCO_3$ added, *centre* and *right* 1 g $CaCO_3$ per pot. *Left* and *centre* uninoculated, *right* inoculated with *Rhizobium trifolii*. **B.** Acid soil. *Left* uninoculated, *right* inoculated with *R. trifolii*.

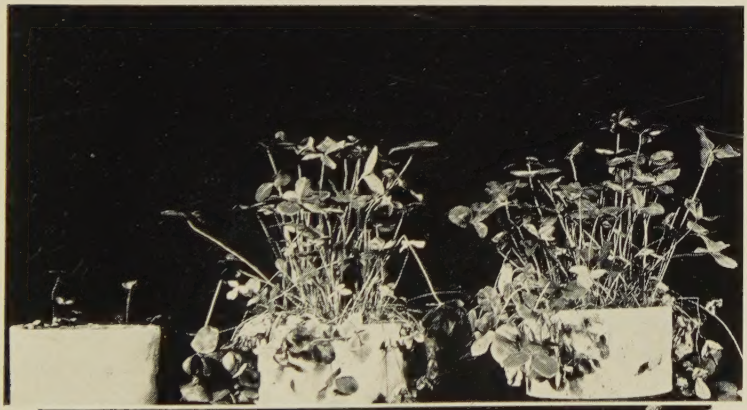
Plate 2. Effect of Rhizobium and yeast extracts on nitrogen fixation and growth of red clover. **A.** Cultures on uninoculated acid soil, (pH 5.1). *Left* control, *centre* and *right* 10 ml per pot of a sterilized suspension of ineffective and effective strains of *Rhizobium trifolii*, respectively. **B.** Uninoculated red clover on acid soil. *Left* (2 pots) control, *right* (2 pots) 2 ml of yeast extract per pot.

A

B

Plate 1

A



B

Plate 2

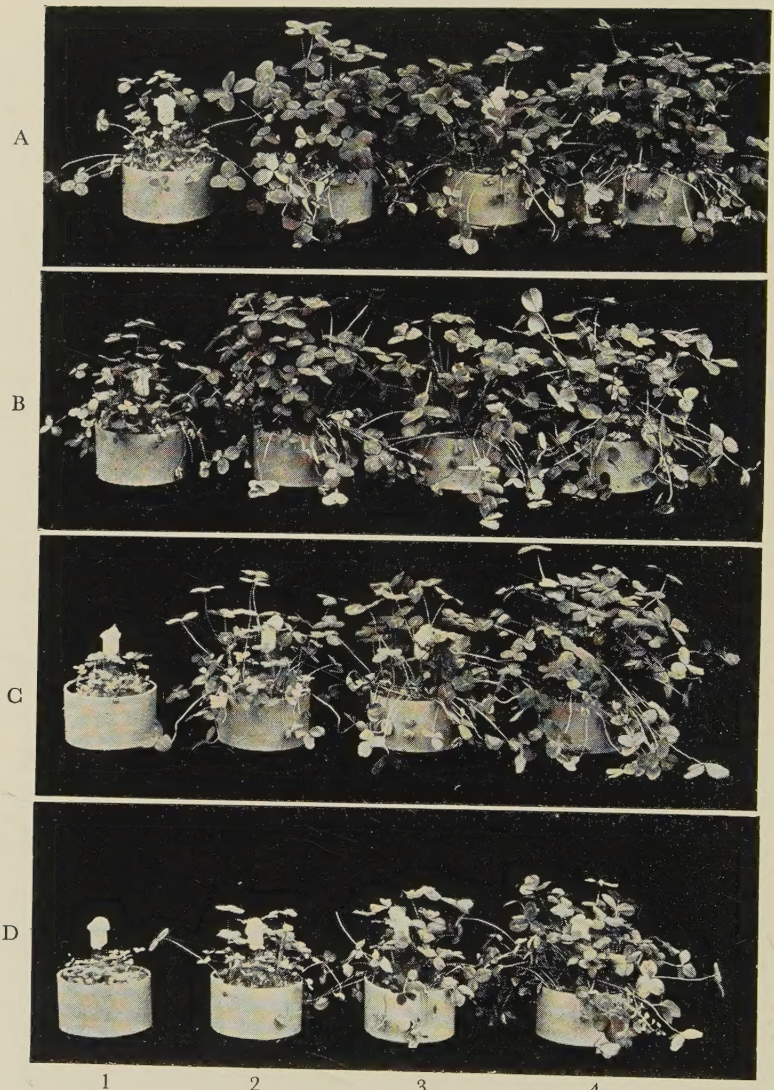


Plate 3

Plate 4

ammonium nitrate gave lower yields than those which had received no combined nitrogen. This was presumably due to the inhibiting effect of ammonium nitrate on nodulation.

A *second* pot experiment was carried out from 18 February to 24 July 1958. The results of this experiment confirmed those of the above one *viz* poorly growing yellow-green clover plants with few small nodules in the control pots and vigorously growing dark-green plants with large numbers of nodules in the pots treated with calcium carbonate (Table 2). The higher yields of the pots with calcium carbonate-treated soil as compared with those of the first experiment were due to the fact that the plants were harvested at a later growing stage.

In a *third* pot experiment which started 18 August and ended 17 November 1958, the effect of added calcium carbonate on nitrogen fixation by red clover was studied with 5 different sandy and peat soils obtained by courtesy of the Institute for Soil Fertility, Groningen. The pH values of these soils varied from 4.3 to 5.0. In this experiment the following treatments were compared: a) control, b) $CaCO_3$, $1\frac{1}{2}$ g per pot in the case of sandy soil and 3 g with peaty soil, c) NH_4NO_3, 120 mg N per pot, d) NH_4NO_3 and $CaCO_3$. One series of pots was left uninoculated, a second series was inoculated with a suspension of an effective strain of *Rhizobium trifolii* which was mixed through the soil. The ammonium nitrate-treated plants were left uninoculated.

The results of this experiment were in agreement with those of the two preceding. On the uninoculated acid soils growth of the red clover was poor with scarcely nodulated yellow-green plants which suffered from nitrogen deficiency. Some plants showed a better appearance due to the presence of a few nodules but there was no tendency of the other plants in the same pot to become nodulated. This was in contrast to the soils treated with calcium carbonate

Plate 3. Effect of calcium carbonate on nitrogen fixation and growth of red clover on four different acid sandy soils. Pots 1 and 3 no calcium carbonate, 2 and 4 with 1.5 g calcium carbonate per pot; 1 and 2 uninoculated, 3 and 4 inoculated with *Rhizobium trifolii* (cf Table 3).

Plate 4. Effect of a suspension of sterilized Rhizobium cells on nitrogen fixation and growth of *inoculated* red clover, growing in culture solution. *Left* 3 pots control, *right* 3 pots 20 ml of Rhizobium extract (containing approximately 8 mg N) per pot.

where all the plants after a lag period of approximately four weeks became adequately nodulated and gave normal green plants (Plate 3 and Table 3).

TABLE 3

Effect of calcium carbonate and ammonium nitrate on yield * (g dry weight per pot) of uninoculated and inoculated red clover, grown in pots on a number of acid soils

Type of soil	Ironstone peat (A)		Sandy (B)		Sandy (C)		Sandy (D)		Sandy (E)	
Organic matter content %	36		7.8		9.5		6.7		2.9	
Treatment	Yield	pH**	Yield	pH**	Yield	pH**	Yield	pH**	Yield	pH**
Uninoculated, control	4.6	5.3	0.9	4.7	1.1	4.3	0.4	4.2	0.2	4.5
Uninoculated + $CaCO_3$	5.4	5.8	3.5	5.1	5.1	5.0	1.2	5.0	1.1	6.1
Inoculated, control	4.1	5.3	2.5	4.5	4.1	4.4	2.7	4.2	1.7	4.2
Inoculated + $CaCO_3$	4.0	6.1	5.2	4.9	4.8	5.1	5.3	4.8	3.4	5.4
Uninoculated + NH_4NO_3	5.4	5.2	3.4	4.5	4.8	4.3	3.1	4.0	1.0	4.5
Uninoculated + NH_4NO_3 and $CaCO_3$	6.6	5.8	6.7	5.0	6.9	5.2	3.9	4.9	2.9	5.7

* Averages of duplicate values.

** At harvest.

Inoculation of the acid soils in general brought about a much improved nodulation as a result of which considerably higher yields were obtained. Inoculation of the calcium carbonate-treated soils in some cases gave also higher yields. In general, nodulation of these plants was still more heavy than when inoculation had been omitted.

Ammonium nitrate gave green plants with relatively high yields. Nodulation of these plants was poor. On calcium carbonate-treated soil ammonium nitrate gave still higher yields. Initially nodulation of the plants was poor, but the young white roots which appeared after the combined nitrogen had been used up contained many small nodules.

From the results of the above three experiments it may be concluded that the poor growth of red clover growing on acid soil is largely due to nitrogen deficiency as a result of an inadequate nodulation. However, infection of the roots and growth and nitrogen-fixing capacity of the nodules are not severely depressed by the low pH of the culture medium. This may be concluded from the heavy nodulation and the relatively high yields of plants, grown on acid soil inoculated with a suspension of *Rhizobium trifolii*. Although the latter result might suggest that living Rhizobium cells are not

present in the acid soils, this suggestion is refuted by the fact that treatment of the soils with sterile calcium carbonate brought about, after a retardation of from three to four weeks, a normal nodulation and nitrogen fixation. Apparently Rhizobium cells were present in small numbers in every acid soil tested, but they were unable to spread in the rhizosphere as they did in the soils treated with calcium carbonate. As a result formation of nodules was absent or restricted to a few localized areas of the root system in the case of acid soil as contrasted to the large numbers of well-distributed nodules occurring on the root system of red clover growing on neutralized soil. It is not known whether this depression of the spread of Rhizobium in acid soil depended on an effect of pH on the growth and the multiplication of the bacteria or on an effect on growth of the plant roots or on excretion by the roots of substances required for growth and multiplication of the bacteria.

c. Effect of various calcium compounds and neutralizing substances on nodulation. To investigate whether the observed effect of calcium carbonate on nodulation and growth of red clover on acid sandy soil was due to its neutralizing effect or to its calcium content, three pot experiments with different calcium compounds and with trisodium phosphate and sodium carbonate, respectively, as the neutralizing agent were carried out. Although trisodium phosphate gave a similar rise in pH as calcium carbonate, it affected nodulation and growth of the clover plants less favourably. The same was true of sodium carbonate. These results might indicate that the observed beneficial effect of calcium carbonate on acid soil was due partly to its calcium content. This conclusion was not corroborated, however, by the fact that a mixture of mono- and dicalcium phosphates which did not alter the pH, did not affect nodulation and plant growth. The same was true of added calcium sulphate. However, application of the latter lowered the pH slightly.

d. Counting of Rhizobium cells in acid soil upon application of calcium carbonate. To study the effect of added calcium carbonate on numbers of Rhizobium cells in acid soil, the following experiment was carried out. Acid sandy soil from the garden plots was transferred to Neubauer glass jars with and without the addition of 2 g of calcium carbonate per 500 g soil. For comparison pots filled with soil from neutral plots were also studied.

Some pots of each series were left unplanted, others were sown on 8 June 1959 with red clover in the usual way. Counts of Rhizobium cells in the soil were made after various periods of time in the way described above. In the case of unplanted soil the same pots were used for every sampling. Of the planted pots two were harvested at each sampling time.

The first counting was made on 18 June when the clover plants were approximately 2 cm high and the roots about 5 cm long. Nodules were not yet visible.

The second counting took place on 1 July. The plants were approximately 5 cm high. Nitrogen deficiency had not yet occurred. The control plants on acid soil had no nodules; two of the twenty plants growing on calcium carbonate-treated soil had a few nodules, the other plants in this pot and also all plants in the second pot of this treatment had no nodules. The plants growing on the neutral soil had from 4 to 8 nodules per plant.

The third counting was carried out on 17 July. The plants growing on the acid soil were approximately 6 cm high; they showed severe symptoms of nitrogen deficiency. Root nodules were absent. The plants of the calcium carbonate-treated soil were slightly taller. They also were severely nitrogen deficient. Some plants showed a darker green leaf colour, however. Many nodules occurred on the roots, but the majority of them were small and white. Plants with green leaves had a greater proportion of pink nodules. The plants growing on the neutral soil were approximately 15 cm high, they had many pink nodules.

The fourth counting was made on 9 August. The control plants growing on acid soil were extremely nitrogen deficient. A few plants of one pot showed a better appearance with green leaves. These plants had a number of pink nodules. In the case of calcium carbonate treatment the red clover had now made vigorous growth, due to the presence of many pink nodules. The yields of dry matter were still lower, however, than those of the clover grown in the neutral soil and the leaf colour was somewhat lighter green.

When the fifth counting took place (27 August) most plants growing in the control pots had died. In one pot of this series two plants had a better appearance. Root nodules were not detected on these plants, however. On the calcium carbonate-treated soil the clover plants had made a vigorous growth and they had a dark-green leaf colour. Large numbers of pink nodules occurred. The neutral soil gave still a somewhat higher yield of clover, presumably due to the earlier start of nitrogen fixation.

The results of the bacteria countings and the yields of tops and roots are given in Tables 4 and 5. It will be seen that no Rhizobium cells were detected in 0.1 g of acid soil. Treatment with calcium carbonate without planting gave approximately one cell per 0.3 to

TABLE 4

ıber of root nodules per pot in red clover and number of *Rhizobium trifolii* cells per 0.1 g of st soil * as affected by calcium carbonate, yeast extract, and planting of the soil with red clover

Soil treatment	Days after sowing									
	10		23		39		60		79	
	Nodules	Cells	Nod.	Cells	Nod.	Cells	Nod.	Cells	Nod.	Cells
soil										
lanted	-	0	–		-	0	–	–	-	0
ted	0	0	0	0	0	0	93	110	0	780
)3, unplanted	–	0	–	–	–	0	-	–	-	1
)3, planted	0	0	2	17	139	490	556	13,000	1,120	28,000
st extract, unplanted	–	0	–	–	-	0	–	–	–	0
st extract, planted	0	0	0	0	5	1	2	0	266	78
tral soil										
lanted	–	49	–	–	–	3	–	–	–	17
nted	0	170	115	330	367	3,300	742	7,900	1,327	4,600

Water content approximately 16 per cent.

TABLE 5

Effect of added calcium carbonate and yeast extract, respectively, on yield * (g dry weight per pot) of red-clover tops

Soil and treatment	pH of soil at 3rd counting	Days after sowing		
		39	60	80
Acid soil	4.7	0.3	0.3	0.3
Acid soil + $CaCO_3$	6.6	0.3	1.4	4.7
Acid soil + yeast extract	4.7	0.4	0.6	1.3
Neutral soil	6.7	0.7	3.0	8.3

* Averages of duplicate values.

0.5 g of soil, a number which did not vary very much in the course of approximately two months. This demonstrates that *Rhizobium trifolii* did not increase in numbers upon liming of the acid soil. In the neutral soil the number of Rhizobium cells was much higher than in the acid soil *viz* about a hundred per gram of soil. This value varied somewhat during the experimental period.

Planting with red clover resulted in an enormous rise in numbers of Rhizobium cells. In the neutral soil this rise started at an earlier date than in the neutralized acid soil, presumably owing to the larger numbers of cells initially present. In the acid soil a distinct increase of bacteria numbers was observed in a few pots.

It must be stressed that the number of Rhizobium cells in the planted neutral and $CaCO_3$-treated acid soil had increased con-

siderably between the first and the second countings *i.e.* during a period in which nodulation was still restricted and liberation of bacteria from the nodules was unlikely to occur. Apparently the increase of the number of bacteria depended on favourable nutritional conditions in the rhizosphere.

e. Effect of number of added Rhizobium cells on nodulation of red clover growing on an acid soil. If it is assumed that the poor nodulation of red clover growing on acid soil is due to an inadequate growth of *Rhizobium trifolii* in the rhizosphere, it may be supposed that relatively large numbers of the symbiotic bacteria have to be introduced into the soil to attain an adequate nodulation. Therefore an experiment was conducted in which the inoculation of the acid soil was made with various dilutions of a suspension of *Rhizobium trifolii*.

The results of this experiment are recorded in Table 6. It will be seen that more than 60,000 bacteria had to be introduced into 500 g of acid soil to attain an optimal nodulation. This was in contrast to the results obtained with sterile clover plants growing on an agar medium in culture tubes, where introduction of one Rhizobium cell resulted in the formation of a normal number of nodules. This demonstrates that conditions for growth and multiplication of the bacteria in the latter case were much more favourable than in the acid soil.

TABLE 6

Effect of number of added Rhizobium cells on nodulation and yield of tops of red clover grown on acid soil

Number of introduced *Rhizobium trifolii* cells.		Tops, g dry weight per pot (duplicates)	Nodulation
Per pot	Per g of soil		
6×10^6	12,000	2.45; 2.50	Many small nodules on most roots
6×10^4	120	1.07; 2.88	,, ,, ,, ,, ,, ,,
6×10^2	1	0.47; 0.89	Few, large nodules, concentrated in a few areas
6	0	0.28; 0.29	No nodules

f. Behaviour of *Rhizobium trifolii* cells added to various soils. To study the effect of pH and organic matter on behaviour of *Rhizobium trifolii* in soil, a dense suspension of this bacterium was added to three different sandy soils from the garden plots of the Laboratory of Microbiology. These soils were the acid

one of pH 5.0 which was employed in the above-mentioned pot experiments, a neutral one of pH 7.5, and a slightly acid one (pH 5.6) which had been treated with stable manure for several years.

At different periods of time after the addition of the bacterium suspension, the numbers of effective Rhizobium cells in the soils were estimated by the above-described technique. The results of these countings are recorded in Table 7. It will be seen that during the first 10 days the numbers of effective Rhizobium cells in the

TABLE 7

Numbers of effective *R. trifolii* cells per 0.2 g of moist soil different periods of time after the addition of a suspension of the bacteria * to the soil

Incubation time	Acid soil		Neutral soil		Soil + stable manure	
	Control	Inoculated	Control	Inoculated	Control	Inoculated
2 hours	—	4.9×10^7	—	—	—	—
1 day	—	2.3×10^8	—	3.3×10^8	—	1.3×10^8
2 days	1	—	3.3×10^3	—	4.6×10^3	—
3 ,,	—	1.7×10^8	—	4.9×10^7	—	4.9×10^7
7 ,,	—	7.9×10^7	—	2.3×10^6	—	7.9×10^7
10 ,,	—	3.3×10^7	—	3.3×10^7	—	1.2×10^7
15 ,,	—	2.3×10^6	—	1.1×10^7	—	2.3×10^6
23 ,,	—	9.5×10^5	—	4.9×10^6	—	2.3×10^6
37 ,,	0	4.9×10^5	1.3×10^4	1.3×10^6	3.3×10^3	1.1×10^6
64 ,,	—	3.3×10^3	—	2.2×10^5	—	2.8×10^5

* 1.3×10^8 cells per 0.2 g soil.

three soils approximated that of the number added. After an incubation time of 15 days considerably lower numbers were counted in all three soils. After 64 days, the acid soil was found to contain a much lower number than the other two soils.

2. *Effect of organic compounds on nitrogen fixation and growth of red clover*

The effect of organic matter on nitrogen fixation by red clover was noticed in the above-described field experiments. Some of the acid plots had been treated with stable manure a few years before starting the experiment. On these plots nodulation and growth of the clover were excellent notwithstanding the fact that the pH was not higher than on similar plots without manurial treatment where no nodules were formed (Table 1).

The results of this observation might be explained in various

ways: 1) The stable manure could have introduced some inorganic factor which promoted in some way nitrogen fixation. 2) It could have introduced some organic component. 3) It could have formed pockets of higher pH in which the bacteria could have survived. 4) It could have improved the carbon dioxide content of the soil. Therefore a number of pot experiments were carried out to elucidate the observed effect of stable manure.

a. Pot experiments with acid soil in Neubauer glass jars

In the *first* experiment the following treatments were compared: 1) Nitrogen, 42 mg per pot, in the form of ammonium nitrate. 2) Casamino acids (Difco), enriched with 20 mg tryptophane per 2.1 g, in amounts equal to 42 mg nitrogen per pot. 3) Concentrated yeast extract, 4.75 ml, containing 42 mg nitrogen, added per pot. 4) Stable manure, 10 g per pot, containing approximately 42 mg nitrogen. 5) Sterilized stable manure, 10 g per pot. 6) Ash of 10 g stable manure incinerated for 4 hours at 300° C. 7) KH_2PO_4, 0.5 g per pot. 8) Sterilized cultures of an effective and an ineffective strain of *Rhizobium trifolii*, respectively. These bacteria were grown as described above. Ten-ml aliquots of suspensions of the bacteria, containing 0.4 mg nitrogen per ml, were added per pot. Two pots of each treatment were left uninoculated, two further pots were inoculated with an effective strain of *Rhizobium trifolii*.

The experiment was started on 28 August and finished on 19 December 1957.

The results are recorded in Table 8. The following conclusions may be drawn from the pots with uninoculated soil: – In the untreated soil the yield of the clover was very low due to nitrogen deficiency. Ammonium nitrate gave an improved growth but the amount applied apparently was too low to produce much higher yields. Nitrogen supplied in the form of amino acids gave slightly better results. In both cases the formation of nodules was poor. Yeast extract gave better yields than was in accordance with its nitrogen content. This was presumably due to the fact that a considerable number of nodules were present on the roots. Stable manure initially did not affect the growth of the plants. At harvest time, however, the leaf colour and the appearance of a number of plants were clearly improved, presumably due to the nodules which had developed. Sterilized stable manure had a less favourable effect whereas ash of stable manure had no effect at all. In the case of phosphate treatment the yields were even lower than those of the

control plants. The most remarkable fact was the favourable effect of dead cultures of effective and ineffective Rhizobium strains. After a retardation period of several weeks, many nodules were formed which brought about a vigorous growth of the plants (Plate 2 A).

TABLE 8

Effect of different organic and inorganic substances on yield ot tops (g dry weight per pot) of uninoculated and inoculated red clover grown on acid soil (Averages of duplicate values)

Treatment	Uninoculated	Inoculated
Control	0.5	3.0
NH_4NO_3 *	0.6	2.0
Casamino acids *	0.9	2.7
Yeast extract *	1.0	1.9
Stable manure *	0.5	2.3
Sterilized stable manure *	0.4	3.0
Incinerated stable manure	0.4	1.4
KH_2PO_4	0.1	2.9
Sterilized effective-Rhizobium extract **	2.5	2.4
Sterilized ineffective-Rhizobium extract **	2.0	2.4

* Corresponding with 42 mg N per pot.
** Corresponding with 4 mg N per pot.

In the case of inoculated soil no clear effect of the various treatments on nodulation and yield of the red clover was observed.

This was in contrast to the results of a *previous* experiment in which stable manure and extract of stable manure were found to have a pronounced effect on yield of inoculated red clover. That experiment was conducted from 28 June to 30 September 1957. Stable manure was applied in amounts of 5, 10, and 20 g per pot, corresponding with 21, 42, and 84 mg N respectively. An extract of stable manure, prepared as described above, gave similar results. Twenty ml of this extract, prepared from 130 g of stable manure and containing 0.75 mg N per ml, was applied per pot. A treatment of 2.5 mg sodium molybdate per pot which was also included in this experiment gave a slightly improved growth. The results of this experiment are given in Table 9. The observed effect of stable manure was not brought about by its nitrogen content. This may be concluded from the fact that a concentrated extract of stable manure, containing only 15 mg N, gave the same results, whereas ammonium nitrate (supplied in amounts of 21, 42, and 84 mg N per pot) depressed nodulation and gave lower clover yields.

TABLE 9

Effect of added stable manure and stable-manure extract on yield (g dry weight per pot) of inoculated red clover, grown on acid soil

Treatment (per pot)	Yield of tops
Control	3.6 *
$Na_2MoO_4.2H_2O$ 2.5 mg	4.4 *
Stable manure, 5g (21 mg N) . .	4.7 **
,, ,, , 10g (42 mg N) . .	5.4 **
,, ,, , 20g (84 mg N) . .	6.3 **
,, ,, extract (15 mg N).	6.0 ***

Average values of the yields of four *, two **, and six *** pots, respectively.

A *third* experiment with sterilized cultures of *Rhizobium trifolii* and with yeast extract added to acid soil was started on 18 February 1958. A treatment with sodium molybdate was also included in this experiment. Each treatment was present in quadruplicate. Inoculation with *Rhizobium trifolii* did not take place and precautions as described above were taken to prevent the contamination of the soil with this bacterium.

Without treatment and in the presence of added molybdate the growth of the clover plants on the acid soil was extremely poor owing to nitrogen deficiency. Treatment with the sterilized Rhizobium suspension and with yeast extract, respectively, gave an improved growth due to the development of root nodules. The response of the plants in the four replications of each treatment was quite irregular, however, as may be seen from Plate 2B and from the yield data in Table 10 where the weight of tops of each individual pot is recorded. This irregularity was also observed in the number of plants per pot which responded to treatment. In general no more than from 2 to 6 of the 20 plants originally present in each pot showed an improved growth and were responsible for the higher yields. No explanation can be given of the irregular response to the added extracts. The beneficial effect of these extracts on nodulation was, however, undoubted (see Table 10).

The effect of added yeast extract on numbers of Rhizobium cells in unplanted and planted acid soil was studied in the experiment described on page 101. The results of the countings are recorded in Table 4 and the corresponding yields of the clover plants in

TABLE 10

Effect of sterilized suspensions of *Rhizobium trifolii* and of yeast extract on yield of tops (g dry weight per pot) and nodulation of red clover, grown on acid sandy soil

Treatment (per pot)		Yield	Nodulation
Control	a *	0.18	Small numbers of small white or brown nodules.
	b	0.05	
	c	0.35	
	d	0.21	
$Na_2MoO_4.2H_2O$, 2.5 mg	a	0.13	Similar to control plants.
	b	0.35	
	c	0.22	
	d	0.32	
Yeast extract, 2 ml	a	9.12	Large numbers of normal nodules.
	b	0.56	
	c	1.10	A few very large composite nodules on the roots of a few plants which had a better appearance.
	d	0.47	
,, 5 ml	a	0.52	Poor.
	b	0.92	Many small white nodules and a few large composite ones on the roots of plants with a better appearance.
	c	0.55	
	d	0.97	
,, 10 ml	a	0.68	Poor.
	b	0.65	
	c	0.60	Many small nodules.
	d	1.30	Many small white nodules and a number of large ones.
Sterilized Rhizobium suspension 5 ml	a	5.70	Large numbers of normal nodules.
	b	0.48	Moderate, with white and brown nodules.
	c	0.48	Poor.
,, 10 ml	a	3.15	Large numbers of normal nodules.
	b	6.90	
	c	0.30	Small numbers of small white and brown nodules.
	d	0.25	

* a, b, c, and d, replicates of one treatment.

Table 5. It will be seen that yeast extract had no influence on the number of bacteria in unplanted soil and a slight effect in the case of acid soil planted with red clover.

b. Effect of various organic compounds on nodulation of inoculated red clover growing in culture solution

Sterile plants of red clover were transplanted on 23 August 1957 to large tubes containing approximately 150 ml of a nutrient medium of the above-mentioned composition. On 12 September the plants were inoculated with an active strain of *Rhizobium trifolii*. Two days later the following substances were added: a) 15 ml of a suspension of killed Rhizobium cells containing 0.42 mg N per ml. b) $3\frac{1}{3}$ ml of conc. yeast extract containing 3 mg N per ml. c) 10

mg nitrogen in the form of amino acids derived from hydrolysed caseine supplied with 1 mg tryptophane per tube. d) 10 mg nitrogen in the form of glutamic acid, aspartic acid and α-alanine, respectively. e) 10 mg nitrogen in the form of ammonium sulphate and potassium nitrate, respectively. Each treatment was present in quadruplicate.

One week after inoculation the first root nodules appeared. Two weeks later pronounced differences in nodulation between different treatments were observed. Plants provided with a suspension of killed Rhizobium cells were well nodulated with large, pink nodules. This contrasted with the untreated control plants where the nodules were similar in number but much smaller and white in colour. The pH values of both series were similar, *viz* approximately 5.9. Added yeast extract gave also relatively large, pink nodules. The pH of the latter solution had increased to 7.6, presumably due to bacterial decomposition of some compound of the yeast extract. In the case of added amino acids, nodulation was more or less depressed.

The results of this experiment indicate that some unknown component present in the Rhizobium or yeast extracts may promote the development of root nodules (Table 11). That amino acids are responsible for this effect is improbable in view of the results obtained with the addition of these compounds.

TABLE 11

Effect of various organic and inorganic compounds on nodulation of red clover grown in culture solution

Treatment	Dry weight of nodules from four different plants, mg per plant
Control	1.1, 1.7, 4.4, 1.4
Sterilized Rhizobium extract *	3.1, 0.7, 4.0, 3.4
Yeast extract **	4.3, 3.0, 4.7, 1.8
Casamino acids **	0.0, 0.3, 0.4, 0.9
Glutamic acid **	0.4, 0.8, 0.7, 0.3
Aspartic acid **	0.2, 0.4, 0.7, 1.3
α-Alanine	0.1, 0.2, 1.0, 0.0
$(NH_4)_2SO_4$ **	0.0, 0.3, 0.3, 0.0
KNO_3 **	0.3, 0.2, 0.5, 0.3

* Corresponding with 6.3 mg N and ** with 10 mg N, respectively.

The beneficial effect of sterilized suspensions of Rhizobium cells on nodulation and growth of red-clover plants was shown in two

further *culture-solution* experiments. Inoculated plants treated with these suspensions were more heavily nodulated and fixed considerably larger amounts of nitrogen than untreated control plants. This was particularly true of plants growing at pH 4.8; at pH 6.8 the differences were less pronounced. Plate 4 shows the effect of Rhizobium extract at pH 4.8. Yield of tops (mean dry weight of 5 plants): control 0.58 g, treatment 1.46 g, and of roots 0.18 and 0.37 g, respectively.

c. Effect of added vitamins on nodulation. To see whether vitamins could affect nodulation of red clover plants, two experiments were carried out.

The *first* experiment lasted from 14 December 1957 to 29 March 1958. Sterile red-clover plants were grown in tubes containing an agar nutrient medium of the above-mentioned composition. A comparison was made between Rhizobium and yeast extracts and a mixture of four vitamins (0.05 to 0.4 ml of a solution containing 10 mg thiamine, 10 mg riboflavin, 10 mg β-alanine, and 4 mg biotin per 100 ml H_2O). Although the plants treated with the extracts and with the vitamin solution tended to be darker green than the control plants, no significant increase in nitrogen content of the plants due to treatment was found.

In a *second* experiment which lasted from 3 April to 28 July 1958, the agar used in the culture tubes had been purified by washing it daily with distilled water for a period of 14 days. The following vitamins were tested singly: thiamine, nicotinic acid, riboflavin, pyridoxine, pantothenic acid, β-alanine, p-amino benzoic acid, and biotin. The vitamins were supplied in amounts of: 10, 20, and 30 μg per tube except biotin which was given in amounts of 4, 8, and 12 μg per tube. The results of this experiment were inconclusive as to the importance of the added vitamins for nodulation.

DISCUSSION

Nodulation of red clover and other leguminous plants, growing on acid soil, in general is poor or absent. As a result development of these plants is restricted and yields are low, mainly due to nitrogen deficiency. It could be suggested that the poor nodulation is a result of the absence of Rhizobium cells or of the inability of either the plant root or the bacteria, or of both, to develop effective

nodules. In the present study inoculation of a number of acid soils with an effective strain of *Rhizobium trifolii* gave well-nodulated red-clover plants which grew vigorously as contrasted with plants growing in uninoculated soil which were poorly nodulated and gave low yields. This shows that the infection of the roots and the formation and functioning of the nodules may be quite normal on such soils, when sufficient numbers of the symbiotic nitrogen fixers are brought into the soil. By using bacterial suspensions of various densities it was found that at least 60,000 Rhizobium cells had to be introduced into 500 g acid soil to obtain adequate numbers of nodules on the clover roots.

Although the results of the inoculation experiments might suggest that no Rhizobium cells occurred in the acid soils, the experiments with calcium carbonate and with various mixtures of organic compounds have shown that *Rhizobium trifolii* was not completely absent from the soils tested. The bacteria may occur in very low numbers, but they increase rapidly when conditions are made more favourable for their multiplication. This may be concluded from the experiments with planted and unplanted soil in which numbers of Rhizobium cells were counted. A pronounced increase took place in planted, acid soil which had been treated with calcium carbonate. Without planting there was no such effect. Planting of acid soil as such did hardly affect the numbers of Rhizobium cells.

From these experiments and from the observation that nodulation, when it occurs at low pH, is restricted to a few, localized areas from which it practically does not extend, it may be concluded that the poor nodulation of red clover growing on acid soils is due to the inability of *Rhizobium trifolii* to grow and spread in the rhizosphere. Whether this depends on a direct effect of pH on multiplication of the bacteria or on an indirect effect, dependant on a limited growth of the plant roots, or on a limited excretion of organic substances required for the growth of the bacteria, is not yet known. The results obtained by Loneragan and Dowling [3] indicate that the critical pH value for the multiplication of *Rhizobium trifolii* in a yeast-extract/mannitol medium lies between 4.5 and 5.0. Vincent [12] found that *Rhizobium trifolii* added to acid soils did not survive when the pH of the soil was below 5. This result was not in agreement with the behaviour of *Rhizobium*

trifolii added to the acid sandy soil employed in the present study. Up to ten days after the addition of a heavy suspension of the bacteria to the acid soil no decline in number of Rhizobium was found. The values reported by Loneragan and Dowling [3] approximate those found for the acid soils used in the present study. This fact could indicate that the inhibited growth of the bacteria in the acid soils is the result of a direct effect of the low pH on the growth of the bacteria. This is not in agreement, however, with the beneficial influence of suspensions of killed Rhizobium cells and of yeast extract on nodulation as it has been observed in the present study with acid soils as well as with culture solutions. Although the possibility of the occurrence of a slight temporary rise in pH following the addition of the extracts to the acid soils can not be excluded, the fact that the effect of these substances was also observed with clover plants growing in culture solutions where the pH was kept at a constant level, renders it improbable that it depended on a change of pH.

The experiments of Albrecht and Davis [1] and of Loneragan and Dowling [3] have demonstrated that the inhibiting effect of a low pH on nodulation might be counteracted to a large extent by increasing the calcium supply of the culture medium. Calcium would function in the formation of nodules and high hydrogen-ion concentrations would depress calcium uptake. In the present study induced calcium deficiency apparently was not responsible for the poor nodulation of red clover in acid media. This may be concluded from the fact that added calcium sulphate, calcium phosphates, and incinerated stable manure had no effect on nodulation. On the other hand neutralization with trisodium phosphate and sodium carbonate worked less favourably than calcium carbonate.

No explanation can be given of the observed effects of the extracts of yeast, sterilized Rhizobium cells, and stable manure. These extracts not only may promote multiplication of *Rhizobium trifolii* and infection of the roots but also the growth of the nodules. The stimulative effect of these substances when added to acid soils is less consistent than that of calcium carbonate, however.

The nature of the component, responsible for the activity of the extracts, is unknown. Experiments with a number of added vitamins (thiamine, nicotinic acid, riboflavin, pyridoxine, pantothenic

acid, β-alanine, p-aminobenzoic acid, and biotin) did not conclusively answer the question whether the effect of the extracts depended on the presence of some vitamin.

The existence of unknown unspecific substances, secreted by plant roots, particularly those of legumes, which may stimulate the growth of Rhizobium strains and nodule initiation is recorded by Nutman [6]. In higher concentrations these substances inhibit nodulation. Treatment of nutrient media containing these inhibitors with charcoal might remove them and result in an improvement of nodulation (Turner [10]). Vantasis and Bond [11], growing pea plants in "used" culture media, found that treatment with charcoal gave lower numbers of pea nodules. Growth of these nodules and nitrogen fixation were considerably improved, however. Rudin [8] obtained more nodules in pea plants treated with extracts of leaves of pea or maize plants than in control plants without this treatment.

SUMMARY

1. Red clover grown on acid soil under field conditions and in pot experiments was found to be poorly nodulated or to lack nodules. As a result of this the plants suffered severely from nitrogen deficiency and gave low yields.

2. Inoculation of the acid soils with a suspension of an effective strain of *Rhizobium trifolii* gave well-nodulated plants which grew vigorously. More than 60,000 Rhizobium cells had to be introduced into 500 g soil to attain a normal nodulation.

3. Calcium carbonate added to the acid soils in amounts of one or two grams per 500 g of soil brought about, after a retardation of approximately four weeks, a normal nodulation of the clover plants. Since precautions had been taken that no foreign Rhizobium cells were introduced into the soil after neutralization, it was assumed that the bacteria had been originally present in the acid soil in low numbers.

By counting the numbers of effective Rhizobium cells, it was found that neutralization of the acid soil without planting of red clover had no effect on the multiplication of the bacteria. In the presence of red clover plants, however, an, enormous rise in *Rhizobium trifolii* was found to occur in neutralized acid soil but not in acid soil. This demonstrates that inability of the bacteria to grow and spread in the rhizosphere presumably is responsible for the poor nodulation of the clover plants on acid soils.

4. Treatment of acid soils with yeast extract or a sterilized suspension of effective or ineffective *Rhizobium trifolii* promoted nodulation of red clover growing on these soils. This effect was less regular than that of added calcium carbonate. Stable manure and stable-manure extract in some cases reacted similarly. So far the substance responsible for the stimulative effect has not been found.

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THE REACTION OF LUCERNE SEEDLINGS TO HIGH CONCENTRATIONS OF MANGANESE

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INTRODUCTION

Previous work of Dessureaux and Ouellette [4] and Dessureaux [3] on manganese toxicity to lucerne has shown that genetic variability exists and that tolerance to manganese toxicity can be increased by selection. In past attempts to select lucerne tolerant to high concentration of manganese, mature plants grown in sand cultures were used, and it was difficult to test sufficiently large populations of plants and their progenies. If it were possible to assess manganese toxicity in the seedlings breeding progress would be quicker and genetical investigations simpler. The present work was undertaken to develop a technique for screening lucerne lines at the seedling stage for tolerance to manganese toxicity.

MATERIAL AND METHODS

Most of the seeds used originated from named varieties. The seeds were scarified to increase germination by gently nicking with a razor blade. Except when specifically stated, seeds were sown in sand at a depth of $\frac{5}{8}$ inch. All experiments were replicated and done in glasshouses.

Three rows of five seeds each were sown in 1-litre amber glass jars or four rows of ten seeds in 1-gallon glazed earthenware pots with a lateral drainage hole. The seedlings, except when otherwise stated, were grown in water-washed silica-sand cultures containing variable amounts of manganese.

In the complete nutrient solution [6] the major elements were supplied from the four following salts: KH_2PO_4, $(CaNO_3)_2.4H_2O$, NH_4NO_3, and $MgSO_4.7H_2O$. Manganese, zinc, and copper were supplied as chloride salts, iron as ferric citrate, boron as boric acid, and molybdenum as ammonium

molybdate. The resulting concentrations in parts per million of the various ions were as follows: 124 for N, 145 for P, 198 for K, 183 for Ca, 49 for Mg, 65 for S, 0.2 for Zn, 0.1 for Cu, 15 for Fe, 0.01 for Mo, and 0.5 for B.

Symptom scores were obtained by rating each seedling from 0 (no symptoms) to 5.

EXPERIMENTAL RESULTS

The effect of various concentrations of manganese

The effect of manganese on germination, measured by the rate of appearance of cotyledons, was investigated by growing in vermiculite fifty seeds each of six varieties of lucerne in six concentrations of manganese. Both manganese chloride and sulphate without the basic nutrient solution were used and the experiment was replicated eight times. Thirteen days after sowing the average numbers of seedlings in the cotyledon stage were:

Mn-concentration	Number of seedlings
Tap water	14.8
10 ppm Mn	16.7
100 ppm Mn	18.0
1,000 ppm Mn	16.7
10,000 ppm Mn	0.1

Manganese concentrations to 100 ppm stimulated germination, and even 1,000 ppm did not delay the appearance of cotyledons. The favourable influence of manganese on the germination of some species has been previously reported by Crochetelle [2]. It is therefore doubtful whether the manganese found in acid soils has any detrimental effect on germination of lucerne.

In another experiment to determine the threshold of injury in young seedlings, two lines were grown in the nutrient solution to which manganese was added in concentrations varying from 0.5 to 1,200 ppm. Seeds were germinated on water-soaked germination pads on 12 December for 5 days, then transferred to 1-litre glass jars at the rate of ten seedlings each. This treatment was replicated four times.

Leaf appearance was delayed when the manganese concentration of the nutrient solution was 100 ppm or more (Table 1), and concentrations of 400 ppm killed 99.9 per cent of the plants in 49 days. The severity of toxicity symptoms increased with increasing

concentrations of manganese. Leaf size measured by the length and breadth of the unifoliate leaf was decreased by 100 ppm, but not

TABLE 1

Effect of manganese concentration on leaf appearance and survival of lucerne seedlings

Manganese concentration ppm	Per cent seedlings		Surviving at 49 days	Number of trifoliate leaves at 56 days
	in unifoliate-leaf stage at 18 days	in first trifoliate-leaf stage at 28 days		
0.5	84	100	100	5.2
25	90	100	100	5.0
100	52	58	62	4.4
400	44	26	0.1	—
800	45	11	0	—
1200	35	30	0	—

by 25 ppm (Table 2). The yield of dry matter 56 days after sowing, based on ten seedlings, was reduced only slightly at 25 ppm, but halved with 100 ppm. These findings were confirmed in further experiments.

TABLE 2

Effect on manganese concentration on toxicity symptoms, unifoliate-leaf size, and dry weight

Manganese concentration ppm	Symptom ratings	Unifoliate leaf		Dry weight (mg)
		Length (mm)	Breadth (mm)	
0.5	0	7.8	11.2	217
25	0.5	8.0	10.8	197
100	3.4	6.4	8.8	113
.....	.....	.....	.....	.....
LSD $P = 0.05$	0.4	0.4	0.8	14
$P = 0.01$	0.4	0.7	1.4	25

The retardation of the appearance of the unifoliate leaf and the decrease in its size by the high concentration of manganese indicated that toxicity can be induced and measured very early in the life-cycle of the lucerne plant.

Differential reaction of lines

A differential reaction of lines to manganese concentration must be demonstrated to establish the value of the technique for selection and breeding purposes. Selfed and crossed progenies of two plants

whose reaction to manganese toxicity was known from past observations were used for this experiment. Plant MT 2 was selected for high tolerance, whereas plant 49-2700 possessed a high degree of susceptibility. The three progenies were grown in manganese concentrations of 0.5 and 100 ppm. In a third treatment, the concentration of the macronutrients was decreased to $\frac{1}{3}$, but the manganese concentration was maintained at 100 ppm, because Williams and Vlamis [8] found that decreased concentration of macronutrients increased the symptoms of manganese toxicity and the manganese content of the plant. The experiment was done in a cool glasshouse and also in one kept at a high temperature. All three progenies were sown together on 15 January, in 1-litre jars at the rate of five seeds each in a split-plot design replicated nine times. The rate of leaf appearance was determined by recording the mean number of days between each of the following successive states: sowing, cotyledon, unifoliate, first and second trifoliate leaves.

The rate of appearance of the cotyledons was not affected by the high concentration of manganese, but the unifoliate leaf and especially the first and second trifoliate leaves unfolded later at the high concentration of manganese. The delay was greater when the concentration of macronutrients was reduced. The interaction, lines × concentration, which expressed differences in the degree of tolerance to manganese toxicity was fairly large for the trifoliate leaves. The delay in the appearance of the first and second trifoliate leaves was greater in the susceptible line than in the tolerant one. Unfortunately, this differential rate of leaf appearance of two lines to various concentrations of manganese was not quite consistent in the two glasshouses. Additional experiments have shown that the rate of leaf appearance is not a sensitive and reliable criterion of tolerance to manganese toxicity.

Two production characters, unifoliate-leaf size and dry weight, were also examined (Table 3). Leaf size was estimated by the length × breadth measurement. Dry weight was determined on the basis of five seedlings when approximately in the 5 to 6 trifoliate leaf stage. The difference in yield between the two glasshouses was due to the difference in date of harvesting 8 April and 21 April in the warm and cold glasshouse respectively. The high concentration of manganese reduced both the size of the unifoliate leaf and the dry weight of the seedlings. This reduction due to manganese

TABLE 3

Manganese content, unifoliate-leaf size, and dry weight of the selfed and crossed progenies of two plants grown in different concentrations of manganese in two glasshouses

Glasshouse and Mn-concentrations	Lines	Unifoliate-leaf size (sq. mm)	(rel.)	Dry weight (mg)	(rel.)	Mn-content (ppm D.W.)
Warmer glasshouse						
0.5 ppm and full strength of macronutrients	MT–2 selfed	188	(100)	490	(100)	78
	49–2700 ,,	120	(100)	387	(100)	76
	MT–2 × 49–2700	161	(100)	512	(100)	75
100 ppm and full strength of macronutrients	MT–2 selfed	124	(66)	205	(42)	409
	49–2700 ,,	63	(52)	75	(20)	434
	MT–2 × 49–2700	103	(64)	175	(34)	420
100 ppm and $\frac{1}{3}$ strength of macronutrients	MT–2 selfed	92	(49)	86	(18)	395
	49–2700 ,,	48	(40)	26	(7)	466
	MT–2 × 49–2700	76	(47)	55	(11)	449
Colder glasshouse						
0.5 ppm and full strength of macronutrients	MT–2 selfed	245	(100)	960	(100)	75
	49–2700 ,,	86	(100)	374	(100)	71
	MT–2 × 49–2700	154	(100)	655	(100)	75
100 ppm and full strength of macronutrients	MT–2 selfed	162	(66)	722	(75)	184
	49–2700 ,,	63	(73)	205	(55)	270
	MT–2 × 49–2700	99	(64)	451	(69)	213
100 ppm and $\frac{1}{3}$ strength of macronutrients	MT–2 selfed	107	(44)	241	(25)	368
	49–2700 ,,	41	(48)	66	(18)	402
	MT–2 × 49–2700	83	(54)	170	(26)	381

toxicity was consistent in the two glasshouses for the size of the unifoliate leaf, but not for dry matter. Increasing the concentration of manganese to 100 ppm decreased dry weight and increased Mn-content to a greater extent in the warmer glasshouse than in the colder one (Table 3).

When the strength of the macronutrients was reduced to $\frac{1}{3}$, the reduction in the unifoliate-leaf size and dry weight was about equal in both glasshouses, but an increase in Mn-content was noted in the colder glasshouse only. It may be concluded that the antagonistic influence of major elements may interact with temperature. This may explain the apparently conflicting results of Löhnis [5], who, using a von der Crone solution containing a lower concen-

tration of manganese, observed more injury in beans at a normal temperature than at a high temperature.

The interaction of lines with manganese concentration, was highly significant for both characters within each glasshouse. The reduction in size of the unifoliate leaf and in dry-matter production of the lines agrees with the increase in manganese content of aerial parts, with the exception of unifoliate-leaf size in the colder glasshouse.

Hence, both unifoliate-leaf size and dry weight of young seedlings can be used to estimate the degree of tolerance or susceptibility of a line to manganese toxicity. Dry-matter production might be the more sensitive criterion, but it is more variable than leaf size. Full strength of the macronutrients and a warm environment helped to discriminate between tolerance and susceptibility, but the greatest difference in manganese content was obtained at $\frac{1}{3}$ strength of macronutrients in the warm glasshouse or at full strength in the cold one.

Maternal influence

Maternal influences may be a source of bias in the assessment of lines for seedling characters. A number of reciprocal crosses were examined for differences in rate of leaf appearance, size of unifoliate leaf, dry weight, and reaction to manganese toxicity. In the present experiment, replicated twelve times, the reciprocal crosses differed in seed weight, therefore it should be possible to determine whether seed size caused any maternal effects. Leaf area in this experiment was measured from blueprints of each unifoliate leaf.

Reciprocal differences occurred in the number of trifoliate leaves, in the area of the unifoliate leaf, and in the production of dry matter (Table 4), and they were generally associated with the weight of the seed. In general, there was good agreement between the two concentrations of manganese for these reciprocal differences in the seedlings characters. Symptom ratings did not exhibit any significant reciprocal differences in the present experiment.

Seed size may account for some of the maternal effects found in reciprocal crosses on seedlings characters such as number of trifoliate leaves, size of the unifoliate leaf, and production of dry matter. However, differences in seed size did not always cause reciprocal differences. Black [1] showed that, in the early vegetative

TABLE 4

Reciprocal differences in some seedling characters in lucerne

Crosses	Weight of 120 seeds (mg)	Symptom ratings	Number of trifoliate leaves		Unifoliate-leaf area		Dry weight	
			0.5 ppm	100 ppm	0.5 ppm (sq. mm)	100 ppm (sq. mm)	0.5 ppm (mg)	100 ppm (mg)
49–2700 × MT–4	309	4.2	6.0	5.2	127	81	1227	358
MT–4 × 49–2700	362	4.0	6.3	5.2	138	98	1415	430
MT–1 × 49–1731	260	3.6	6.2	5.0	95	76	928	300
49–1731 × MT–1	340	3.3	5.7	5.1	100	82	850	336
MT–8 × MT–10	248	2.6	4.6	3.5	97	66	476	166
MT–10 × MT–8	368	2.7	5.2	4.4	118	40	662	294
MT–8 × MT–11	250	2.8	4.6	3.7	118	76	536	198
MT–11 × MT–8	365	2.7	5.1	3.8	154	113	852	271
.....	.....	.....	.....	.....	.....	.....	.....	.....
LSD $P = 0.05$	–	0.3	0.4		16	12	146	65
$P = 0.01$	–	0.5	0.5		21	15	195	86

phase of subterranean clover the effect of seed size was such that it eliminated strain differences when common embryo weights were used; strain differences, however, still existed in lucerne when seed weights were equal. The reaction of seedlings to manganese toxicity, whether measured by symptom ratings, per cent reduction of the size of the unifoliate leaf, or by per cent reduction of dry-matter yield only occasionally showed reciprocal differences, indicating that the effect of seed size is not likely to be affected greatly.

CONCLUSIONS

The induction of manganese injury in the early vegetative growth of lucerne, and the differential response of various lines to manganese concentrations of 0.5 to 100 ppm establish the feasibility of using a screening technique in the seedling stage. It is interesting to note that the screening of young seedlings of lucerne for adaptation to low pH (3.5) has been used by Schander [7] as a technique to produce strains tolerant to acid soils.

The criteria which may be used with some confidence as a measure of tolerance or susceptibility to manganese toxicity are symptom ratings, manganese content, per cent reduction in unifoliate-leaf size and in dry matter. Symptom ratings, however, have the disadvantage of being dependent upon the subjective influence of the observer, and therefore the readings can be rather variable, whereas unbiased and precise results are required, and they may not be sufficiently accurate to detect small differences. Manganese content gives a quantitative assessment which is very useful, but the technique is rather laborious. It is possible, however, that a more complete picture of the degree of tolerance would be obtained if the manganese content of the roots were also determined. The reduction in unifoliate-leaf size and dry matter observed in 100 ppm of manganese as compared with 0.5 ppm, gives a measure of tolerance which generally agrees with either manganese content or symptom rating or both. Reduction of dry matter may be more efficient, but more variable than reduction in the size of the unifoliate leaf in detecting differences in the degree of tolerance.

Evidence of maternal influences from reciprocal crosses tends to indicate that seed size in lucerne is not likely to offset to any great extent the reaction of the young seedlings to manganese toxicity. Testing and selection of lucerne in the seedling stage should be a powerful tool in the development of varieties tolerant to manganese toxicity, and might be a useful technique in studying the inheritance of that character.

SUMMARY

Concentrations of manganese lower than 100 ppm did not induce detectable differential effects in young seedlings of lucerne. Concentrations of 400 ppm or higher were unnecessarily high for inducing differential responses.

Germination was not affected by concentrations of less than 1,000 ppm Mn.

Hundred ppm Mn retarded the rate of leaf appearance, reduced the size of the unifoliate leaf, decreased the production of dry matter, and induced toxicity symptoms.

There were no clear-cut differences between lines in the effects of manganese concentration on the rate of leaf appearance, so this character is not a reliable criterion of tolerance to manganese toxicity. However, reduction in size of the unifoliate leaf and decrease in dry weight by high concentration of Mn differentiated clearly between seedlings in their reaction to toxicity. Symptom ratings and manganese content of the shoot gave supplementary information.

Maternal influences were unimportant in assessing the reaction of lucerne seedlings to manganese toxicity, but seed size may sometimes affect characters used to measure the degree of tolerance or susceptibility.

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CATION-EXCHANGE CAPACITY AND PECTIN GRADIENTS IN LEEK ROOT SEGMENTS

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INTRODUCTION

Certain exploratory studies on the relationship between cation-exchange capacity (C.E.C.) and pectin content of plant roots were carried out using leek roots, which have a uniform cross-section over much of their length, making it possible to experiment with whole roots or cut segments of known surface area. Although the main experiments were ultimately carried out with disks of storage tissue [2], quantitative changes in C.E.C. and pectin along the root are of interest and do not appear to have been reported previously.

Mattson and his co-workers [7] have shown by staining methods with methylene blue that the most active acidoids of the root are localised in the surface layer and most of all in the growing tip. It was also pointed out that the root acidoid content could be attributed to pectic substances, and that these can function as cation exchangers. More recently, using ether-killed plant roots, Keller and Deuel [5] were able to demonstrate that 70 to 90 per cent of their C.E.C. was due to their pectic content. We have found that for disks of sugar beet tissue the figure approaches 100 per cent.

A number of workers have studied the root and its differentiating zones in relation to cell number and size, respiration, salt accumulation and translocation and water uptake. Cells are most numerous and have the highest oxygen consumption 1 to 2 mm from the tip. Beyond 5 mm from the tip, the number of cells per mm of length remains practically constant although wet weight is increasing and dry weight decreasing in step with elongation and vacuolation [8]. Total nitrogen and protein nitrogen per unit length are highest 2 mm from the root tip [6]. In the light of these known changes it

seemed likely that C.E.C. would also change with distance from the root apex.

Kramer and his co-workers [6] have made extensive investigations of changes in mineral content along the root. In general maximum accumulation does not always imply maximum translocation from that zone. Fuller [3] examined changes in organic constituents including pectic substances of the cell wall in cells at three stages of development along the root. Hanson and Bonner [4] reported an increase in C.E.C. of tissue following pretreatment with indolyl acetic acid, while Wilson and Skoog [10] found that the stimulation of cell expansion by IAA results in an increase in pectic substances in the tissue.

METHODS

Sections were cut by hand of roots (approximately 1 mm in cross-section) from leek plants (*Allium ampeloprasum*) grown in soil. The length of the sections varied as follows: –

Experiment 1. Segments of length 2.5, 2.5, 5, 10, then 20 mm until a distance 140 mm from the apex was reached.

Experiment 2. The 30 mm length from 50 to 80 mm from the root tip was divided into segments 10 mm, 5 × 2 mm, and 10 mm long.

Sections were combined to give enough material for measurement of cation-exchange capacity, mineral content, pectin and total N in the first experiment. For example, 250 segments of 2.5 mm length were required for each C.E.C. measurement. In the second experiment C.E.C. only was determined.

C.E.C.

The root sections were acid washed in 0.01 *N* HCl, washed free from acid and titrated to pH 7 in *N* KCl using 0.01 *N* KOH over a 5-minute period [1].

Pectin

The micro-method of Tracey [9] for total uronic acids was used, in which CO_2 evolved on decarboxylation by 12 per cent (w/w) HCl is measured in a Van Slyke apparatus. The results were then calculated as me H per 100 g dry roots by dividing the weight of CO_2 evolved by 44, assuming all the CO_2 was derived from pectic substances.

Mineral analysis.

The sample after being oven dried was ashed at 450°C, extracted in 1 per cent HCl and analysed spectrochemically for K, Na, Mg, and Ca.

Kjeldahl nitrogen.

Micro-digestion of dry matter followed by steam distillation in the Markham apparatus.

Respiration.

Oxygen uptake was determined manometrically at 25°C over a 3-hour period. Distilled water was used as the experimental fluid.

RESULTS AND DISCUSSION

The results obtained are given in Tables 1 and 2. In Table 1 the C.E.C. has been expressed both on a dry-weight and a surface-area basis. In both cases the C.E.C. is highest in the first segment (0 to 2.5 mm) and decreases with increasing distance from the root tip.

TABLE 1

C.E.C., pectin content, respiration, and mineral content of leek roots

stance n apex mm)	C.E.C. me/100 g dry roots	C.E.C. me/sq.m	Pectin me/100 g dry roots	Respiration ml O_2/100 g dry wt/hour	Mineral content (percentage in dry matter) N	K	Ca	Mg	Na
0 2.5	134	11.9	63	212	2.17	3.10	0.33	0.26	0.50
5	120	10.1	56	201	2.15	3.11	0.28	0.27	0.52
10	57	5.8	53	152	2.06	3.66	0.24	0.27	0.48
0– 20	65	6.0	51	160	2.06	5.00	0.24	0.28	0.66
0– 40	39	3.6	50	130	1.96	4.66	0.21	0.28	0.55
0– 60	28	3.2	48	119	1.92	4.17	0.21	0.29	0.48
0– 80	26	3.4	48	116	—	4.42	0.21	0.28	0.48
0–100	30	4.1	45	110	1.76	4.50	0.24	0.28	0.46
0–120	33	5.1	40	94	1.65	4.50	0.23	—	0.44
0–140	46	7.3	36	80	1.64	4.41	0.21	0.23	0.40
le roots	26	5.0	36	—			—	—	—

This, together with the similar trends in respiration and nitrogen contents, is in keeping with the known fact that the tip is the most metabolically active zone in the root. There is an increase in K content with distance from the tip but no considerable change occurs in Ca, Mg, or Na along the root.

The results of the second experiment given in Table 2, compare results for C.E.C. on a dry-weight and surface-area basis. The subsectioning of the 10-mm segments into 5 × 2 mm pieces is accompanied by a decrease in dry weight, presumably due to the loss of some cell contents, and an increase in titration value. This results in an apparent increase in the C.E.C. of this zone, when compared with the two adjacent zones. However, when calculated on a surface-area basis, all three zones are found to have similar exchange ca-

pacities. This suggests that the high values for the 0 to 5 mm zone in the first experiment are valid, since even on a surface-area basis they are higher than in the rest of the segments. It is interesting to note that the respiration of onion roots is the same irrespective of whether measured in a 5-mm segment or 5 × 1 mm segments [8].

TABLE 2

Comparison between C.E.C. of samples of 3 adjacent 10-mm segments the centre one being divided into 5 × 2 mm segments

Distance from apex (mm)	Titre ml 0.01 *N* KOH	Dry weight (mg)	C.E.C.	
			me/100 g dry roots	me/sq.m
50—60 A	1.97	59.4	33.2	5.58
B	1.85	61.3	30.2	5.25
60—70 A	2.41	36.8	67.4	5.74
B	2.36	38.1	61.9	5.62
70—80 A	1.64	61.4	26.8	4.65
B	1.93	64.2	30.1	5.48

Table 1 shows that pectin is highest at the root tip and decreases steadily with distance from the tip. It thus follows the same pattern as C.E.C., respiration, and nitrogen content.

However, when we come to consider a correlation between C.E.C. and pectin along the root, while there is good qualitative agreement for regions other than the first 20 mm, pectin is higher than C.E.C. Subsequent work has shown that a deduction has to be made for methyl pectin since it is not considered to take part in exchange processes [5]. In order to obtain a correlation between C.E.C. and pectin in our work with sugar-beet disks we have found it necessary to boil the disks in water for 5 minutes to remove substances yielding CO_2 on decarboxylation yet not contributing to C.E.C.[2].

For segments nearer the root tip where C.E.C. is higher than pectin it is probable that C.E.C. values are over estimated on the dry-weight basis due to the loss of cell contents on sectioning.

Although root segments were shown to be suitable for studies on the relationship of C.E.C. to other factors, the main experiments were carried out with disks of storage tissue, in which similar changes were found, and where the technical difficulties were fewer.

SUMMARY

Cation-exchange capacity (C.E.C.), pectin, respiration, and nitrogen content have been measured in successive segments of leek roots over a distance of 140 mm from the root tip. All are highest at the root tip and decrease with increasing distance from the root tip.

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THE EFFECT OF FERROUS SULPHATE ON THE YIELD AND MANGANESE UPTAKE OF OATS ON SANDY SOIL FERTILIZED WITH NATURAL PYROLUSITE

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Previous investigations (Boken [1 2]) have shown that fertilization with ferrous sulphate for oats and beets on various soils had the same effects as fertilization with manganese sulphate, presumably on account of the ability of ferrous sulphate to reduce manganese higher oxides in the soil to manganese compounds available to the plants.

In pot experiments (Boken [3]) where oats were given commercially produced pyrolusite with and without ferrous sulphate, a considerably higher yield and manganese uptake per pot were found when the soil was fertilized with pyrolusite as well as with ferrous sulphate as compared with soil fertilized with pyrolusite only. The pot experiments described below investigated whether a similar effect of ferrous sulphate could be found when natural pyrolusite was simultaneously given to the soil.

METHODS AND MATERIALS

The pot experiments were carried out in cylindrical zinc pots coated on the inside with paraffin wax. The pots were fitted with external pipes for watering from the bottom of the container; broken flints were used as drainage material. The capacity of the pots were 20 l, and their surface area 500 sq. cm.

Measurement of pH was made with the glass electrode.

The organic carbon and *calcium carbonate content* of the soil was determined by using the Ter Meulen and the Scheibler apparatus, respectively.

The manganese content of crops was determined colorimetrically as per-

manganate after wet ashing with a mixture of sulphuric and perchloric acids. Divalent manganese was oxidized in the neutralized solution by potassium persulphate in the presence of sulphuric acid and silver ions.

The total manganese content of the soil was determined by refluxing 20 g of air-dried soil with 200 ml 20% hydrochloric acid for one hour, and the content of manganese then determined as above.

The exchangeable manganese of the soil was estimated by shaking for one hour 20 g soil with 200 ml *M* magnesium nitrate. *The content of reducible manganese in the soil* was determined by shaking another 20 g of soil with 200 ml *M* magnesium nitrate containing 5 or 10 mg hydroquinone. The difference between the amounts of manganese found by shaking the soil with magnesium nitrate alone and magnesium nitrate + hydroquinone is denoted as reducible manganese.

The X-ray analyses of pyrolusite were made with chromium radiation and a vanadium filter in a 19-cm Bradley camera.

Active MnO_2 in pyrolusite was determined by reduction with an excess of sodium oxalate in dilute sulphuric acid followed by titration of the excess sodium oxalate with permanganate.

Treatments

The pot experiment was made with a sandy soil from field D_4 of the State Experimental Station at Store Jyndevad in South Jutland. The soil had a pH of 7.7, an organic carbon content of 1.91 per cent and a calcium carbonate content of 0.4 per cent. Furthermore, the soil contained 1.2 ppm exchangeable manganese, 7.8 ppm reducible manganese when treated with 5 mg hydroquinone and 11.8 ppm with 10 mg hydroquinone. The total manganese content of the soil was 270 ppm. Thus, it was a soil with so low a content of exchangeable manganese that, without addition of manganese, it should be expected to give crops deficient in manganese.

As a basal dressing for the experiment the following was used per pot: 20 g $NaNO_3$, 12 g KH_2PO_4, 6 g K_2HPO_4, 20 g $MgSO_4.7H_2O$, 0.5 g $CuSO_4.5H_2O$, and 0.1 g $Na_2B_4O_7.10H_2O$. All salts were Merck's A.R., and were added in solution.

The experimental fertilizers were $MnSO_4.4H_2O$ and $FeSO_4.7H_2O$, both Merck A.R.; furthermore, hydroquinone (May & Baker suitable for analysis) and finally a natural pyrolusite of unknown origin supplied by "Mineral Unlimited", U.S.A.

The pyrolusite was ground in an agate mortar, and by means of a JEL set of sieves a fraction was produced with a particle diameter of less than 50μ. It contained 31.2 per cent of active MnO_2, calculated as Mn 19.7 per cent. The pyrolusite fertilizer contained no exchangeable manganese as determined by shaking for one hour with *M* magnesium nitrate. Furthermore, it was subjected to an X-ray crystallographic examination; the X-ray data are listed in Table 1.

From Table 1 it will be seen that, besides containing β-manganese, the

TABLE 1

Natural pyrolusite. X-ray data					
d	I	Phase	d	I	Phase
4.25	2	Q	2.04	2	u
4.06	2	F	1.98	2	β and Q
3.66	1	F	1.90	2	u
3.34	9	Q	1.82	5	Q
3.11	1	β and F	1.71	1	u
2.70	1	M	1.69	1	u
2.55	2	F	1.67	3	Q
2.52	1	F	1.66	3	M
2.45	2	Q	1.62	2	β
2.43	2	β	1.54	5	β and Q
2.34	2	M	1.49	1	u
2.28	2	Q	1.47	2	u
2.23	2	β	1.45	2	Q and M
2.14	1	β and Q	1.43	1	β and M

d = interplanar spacings in Ångstrøm units
I = estimated relative intensities
F = feldspars
M = manganite
Q = quarts
u = unindentified

natural pyrolusite also contained manganite, and that quartz and feldspar were found among the impurities.

The experimental design is shown in Table 2, and 5 replications were used. The experimental fertilizers were uniformly mixed with the whole quantity of soil in the pot.

To the soil were added equivalent quantities of hydroquinone and ferrous sulphate, 5 g of hydroquinone and 25 g of ferrous sulphate, respectively, being added per pot and 20 kg of soil. Experiments with different soils, including the soil from Store Jyndevad, have shown that if more than 5 g of hydroquinone per pot is applied, a toxic effect is seen, resulting in reduced crop yield. Five grams of hydroquinone per 20 kg of soil corresponds to 5 mg of hydroquinone per 20 g of soil, this quantity having been used for determining the content of reducible manganese in the soil.

The test crop was oats, var. Victory; the pots were kept in a greenhouse during rainy weather, and the soil was maintained at a moisture content equal to 50 percent of its total waterholding capacity calculated on an air-dry basis.

EXPERIMENTAL RESULTS

The oats were sown on 17th April 1956 and germinated uniformly, germination being completed on 3rd May. On 23rd May the plants were thinned to 25 plants per pot. In the last week of May and the

first week of June the first symptoms of manganese deficiency were observed in the oats in the order of succession in which the experimental treatments are mentioned here: Control, 0.1 g Mn, 100 g pyrolusite, 0.2 g Mn, 25 g ferrous sulphate, 0.3 g Mn, 5 g hydroquinone, 0.4 g Mn. In the pots with pyrolusite + ferrous sulphate or hydroquinone no symptoms of manganese deficiency were seen in the oats.

The oats began earing from 23rd till 26th June, and all plants had finished earing on 4th July. On 16th August the oats were ripe and were harvested.

On 15th July the growth of the oats was assessed according to an arbitrary scale, 1 being the poorest growth (severe manganese deficiency), 2 representing medium manganese deficiency, and 3 no symptoms of manganese deficiency. Table 2 shows the average scores for each treatment.

TABLE 2

Assesment of growth of oats. July 15th. 1956	
Treatment per pot	Mean scores *
Control	1.0
0.1 g Mn as manganese sulphate	1.8
0.2 g „ „ „ „	2.0
0.3 g „ „ „ „	2.6
0.4 g „ „ „ „	2.8
100 g natural pyrolusite	2.6
5 g hydroquinone	2.8
25 g ferrous sulphate	2.4
100 g natural pyrolusite + 5 g hydroquinone	3.0
100 g natural pyrolusite + 25 g ferrous sulphate	3.0

* 1 = severe manganese deficiency
2 = medium „ „
3 = no „ „

If the estimated values in Tabel 2 are compared with the figures showing the yield of dry matter determined one month later and shown in Table 3, it will be seen that the results gained by the two methods are in agreement as a low value in Table 2 corresponds to a small yield of dry matter in Table 3 and *vice versa*. Table 3 also shows the content of manganese in the crop and the pH in the soil determined after harvest.

TABLE 3

Pot experiments 1956. Oats					
Treatment per pot	g dry matter per pot. Grain + straw		Mn in dry matter ppm	Uptake of Mn mg per pot	pH of soil 11/9, 1956
	Yield	Increase in yield over control			
Control	43.2	—	22.9	0.99	7.6
0.1 g Mn as manganese sulphate	61.8	18.6	18.9	1.17	7.7
0.2 g ,, ,, ,, ,,	74.6	31.4	18.9	1.41	7.7
0.3 g ,, ,, ,, ,,	95.3	52.1	21.1	2.01	7.8
0.4 g ,, ,, ,, ,,	103.9	60.7	23.1	2.40	7.7
100 g natural pyrolusite	85.1	41.9	20.3	1.73	7.7
5 g hydroquinone	99.4	56.2	40.8	4.06	7.8
25 g ferrous sulphate	88.5	45.3	19.3	1.71	7.6
100 g natural pyrolusite + 5 g hydroquinone	121.9	78.7	50.0	6.10	7.7
100 g natural pyrolusite + 25 g ferrous sulphate	106.8	63.6	46.8	5.00	7.6
S.E. ±	4.0	5.2			

DISCUSSION AND CONCLUSIONS

The soil used for the pot experiments containing 1.2 ppm exchangeable manganese was shown to be deficient in manganese as the symptoms of manganese deficiency were extensive in the oats given only the basal dressing and decreased in intensity and frequency with increasing supplies of manganese sulphate (Table 2). Furthermore, the yield of dry matter increased with application of increasing quantities of manganese sulphate (Table 3). The relative manganese content of the oats, after an initial decrease in value, increased with additional manganese supplies (Steenbjerg [8]).

The reducible manganese of the soil was assessed in the laboratory by addition of 5 and 10 mg of hydroquinone, respectively, per 20 g of soil corresponding to 5 and 10 g of hydroquinone per pot. Where 5 mg of hydroquinone was used, 7.8 ppm reducible manganese was found while 10 mg gave 11.2 ppm. If it is desired to determine the quantity of a nutrient available to the plants under conditions of growth as close as possible to those existing in the field, it would, in such case, be preferable to use 5 mg of hydroquinone per 20 g of soil as harmful effects have appeared in the crop where higher concentrations of hydroquinone have been used for pot experiments.

Furthermore, where manganese minerals are insufficiently available to the plants for the maintenance of vigorous growth, it must, in principle, be preferred to use the mildest possible procedure in the laboratory for the assesment of the available manganese content of the mineral as a too drastic laboratory treatment might dissolve quantities of manganese that have no relation to the ability of plants to absorb manganese from the minerals in question (Jones and Leeper [5]).

The 7.8 ppm of reducible manganese found in the soil sample appeared to be important for the growth of the plants when reducing agents were added to this soil since an increase in the yield of dry matter of 56.2 and 45.3 g was found when 5 g of hydroquinone and 25 g of ferrous sulphate, respectively, were applied per pot.

If the laboratory method used here for determining reducible manganese in the soil is to be useful in agricultural practice for determining the amount of manganese that can be made available to the plants during a period of growth, then, of course, the effect of the reduction processes in the field must be comparable to the effect obtained by adding hydroquinone or ferrous sulphate to the soil in the pot experiments.

Table 1 shows that impure pyrolusite, which also contained some manganite, was used in the pot experiments. Therefore, it was of interest to investigate whether the pyrolusite contained substances, other than manganese, that might have either a growth-stimulating or growth-retarding effect. This was done in a system of co-ordinates (Steenbjerg [7]) by comparing the relationship between the production of dry matter and the quantity of manganese absorbed per pot when pyrolusite and manganese sulphate, respectively, were applied to the soil, as shown in Fig. 1, where the fully drawn part of the curve represents the relationship between dry-matter production and the quantity of manganese absorbed.

It will be seen that, for pyrolusite, the point showing the relationship between the dry-matter production and the total absorbtion of manganese is situated on the curve describing the same relationship for manganese sulphate. This investigation indicates that the pyrolusite used did not contain substances, beside manganese, of importance for the growth of the plants under the given conditions of growth. If the point for pyrolusite showing the relationship of

the dry-matter production to the total absorbtion of manganese had been outside the curve showing the same relationship for manganese sulphate, it should be considered whether such deviation was caused by the manganese in the two fertilizers being available to the plants at different times during the period of growth, or whether another nutrient had been supplied in growth-stimulating or growth-retarding quantities.

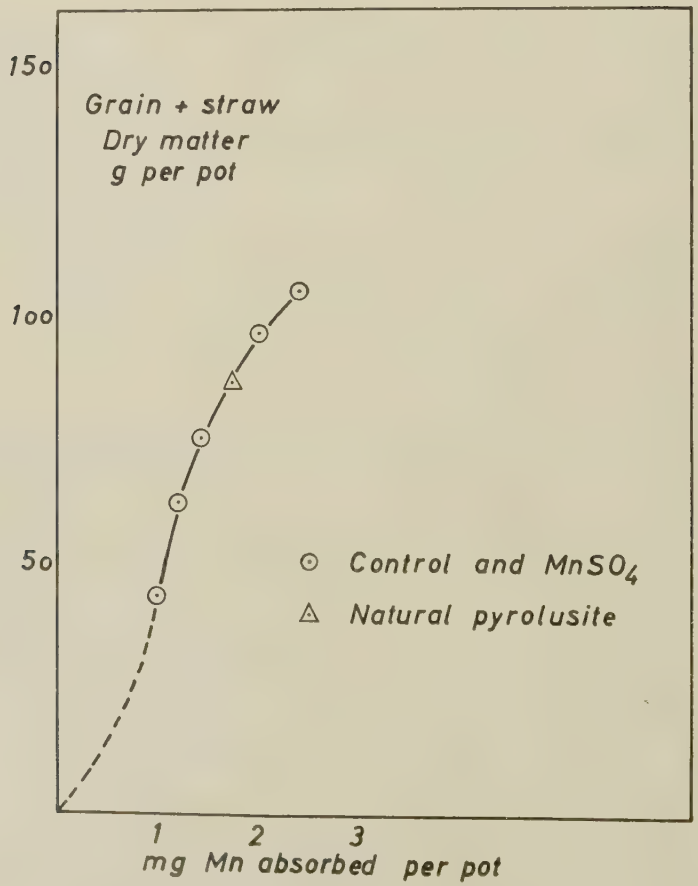

Fig. 1. Relationship between dry-matter production and quantity of manganese absorbed.

Table 3 shows that 100 g of natural pyrolusite per pot gave a yield that was nearly double the yield of the control and, likewise,

the manganese uptake per pot was about 75 per cent higher in the pots supplied with pyrolusite than in the pots given the basal dressing.

When the effects of pyrolusite were compared with the effects of applied manganese sulphate, the dry-matter yield and the manganese uptake per pot were found to be of the same magnitude when fertilized with 100 g of pyrolusite + 50 μ (19.7 g Mn) per pot as when fertilized with 0.2 to 0.3 g of manganese as manganese sulphate. Thus in order to obtain a yield of 85 g of dry matter per pot about 75 times as much manganese in this finely ground pyrolusite must be used as compared with manganese as manganese sulphate.

An application of equivalent quantities of hydroquinone and ferrous sulphate gave a yield of dry matter that was slightly above double the yield of the control. However, an application of 5 g of hydroquinone per pot gave a higher yield and a considerably larger uptake of manganese than an application of 25 g of ferrous sulphate. The explanation of this may be that hydroquinone, which dissolves more slowly than ferrous sulphate, is also more slowly oxidized than ferrous sulphate and, therefore, is able to supply the plants with manganese during a longer period than ferrous sulphate and may be also at a time when an adequate supply of manganese is of decisive importance for the manganese nutrition and development of the plant. As hydroquinone is probably oxidized to quinone in the soil and as Steenbjerg [6], in pot experiments with oats, has found a manganese effect of quinone applied to a podzolized sandy soil, the possibility of a similar quinone effect in the experiments described above cannot be ignored.

Where 100 g pyrolusite + 50 μ per pot was added together with either hydroquinone or ferrous sulphate, the soil supplied with hydroquinone showed an increase in the yield of dry matter of 23 per cent and an increase in the uptake of manganese per pot of 50 per cent; soil supplied with an equivalent quantity of ferrous sulphate showed an increase in the yield of dry matter of 21 per cent and an increase in the manganese uptake of 192 per cent. Hence, when equivalent quantities of hydroquinone or ferrous sulphate are applied, the addition of the above-mentioned natural pyrolusite seems to have the same effect on the yield of dry matter of oats.

Furthermore, the last column in Table 3 shows that the pH of

the soil determined after the harvest varied but slightly in the various treatments and that pH values of the same magnitude were found in the pots supplied with basal dressing and those supplied with ferrous sulphate (Bromfield [4]).

SUMMARY

The effects of soil treatments with ferrous sulphate and an equivalent amount of hydroquinone on the yield of dry matter and the manganese uptake of oats were investigated in pot experiments using a sandy soil deficient in manganese, with and without additions of finely ground, natural pyrolusite containing some manganite.

In soil not fertilized with pyrolusite an application of hydroquinone gave a higher yield of dry matter and a considerably higher manganese uptake than an application of the equivalent quantity of ferrous sulphate.

In soil fertilized with hydroquinone an increase of 23 per cent in the yield of dry matter and an increase of 50 per cent in the manganese uptake per pot were found when the soil was fertilized with 100 g of natural pyrolusite $< 50\mu$ per pot. In soil fertilized with a quantity of ferrous sulphate equivalent to hydroquinone, an increase of 21 percent in the yield of dry matter and an increase of about 200 per cent in the manganese uptake per pot were found when the 100 g of pyrolusite was applied.

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THE SYMBIOTIC PERFORMANCE OF LUPIN BACTERIA UNDER GLASSHOUSE AND FIELD CONDITIONS

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INTRODUCTION

In Western Australia *Lupinus angustifolius* L. is used as a green-manure crop, and *L. digitatus* Forsk. for grazing. *L. luteus* L. is considered by Gladstones [7] to be of potential value as a pioneer crop for some of the virgin soils which are being improved.

Poor nodulation is common in new stands of lupins, but the species *L. angustifolius* and *L. luteus* have been observed to nodulate poorly even in areas where *L. digitatus* is easily established. These observations suggested the possibility that there is marked host-strain specificity between the genus *Lupinus* and its nodule bacteria, as has been found in clovers and medics by Vincent [11]. Bacterial strain has been reported by Cass-Smith [4] as a factor in the establishment of *L. angustifolius*, and host strain specificity in lupins has been reported by Fedorov and Svitych [6].

On the other hand, *L. digitatus* has become naturalised over a wide area of sandy coastal soils in Western Australia, suggesting that it had a symbiotic affinity with the nodule bacteria of some indigenous legume(s). This idea conflicts with that embodying host-strain specificity within the genus *Lupinus* itself, since there are no closely related legumes in the south-western Australian flora.

The work recorded here was undertaken to (i) obtain a range of nodule bacteria from lupins naturalised in the local environment; (ii) define the symbiotic interaction of these rhizobia with their hosts of origin, and select bacterial strains capable of effective

symbiosis with lupins in the field; (iii) examine the ability of the three lupin species to enter into symbiosis with "indigenous" rhizobia. Phase (iii) is continuing.

ISOLATION OF RHIZOBIA *

Two hundred and sixteen platings on yeast–sugar agar were made from nodules of naturalised *L. angustifolius*, *L. digitatus*, and *L. luteus*, and plates were incubated at 27°C. Daily inspections of the plates were made, and observations on colony characters and order of appearance recorded.

At three days, fast-growing colonies were present on all plates. During the period 3 to 10 days, no colonies appeared which were not visible at three days. At 12 days minute colonies were observed on 11 of the plates and more appeared until at 21 days slow-growing colonies were present on approximately half the plates. Some of these failed to grow on sub-culture.

Thirty-six cultures representing the fast-growing population and 11 cultures from the slow-growing population were selected and used in a pot experiment to determine the infective ability of each culture upon the lupin species from which it was isolated.

Without exception *infective* pure cultures exhibited slow growth on yeast–sugar media. This supports the findings of Eckhardt, Baldwin and Fred [5] who resolved conflicting reports on the infectiveness of fast- and slow–growing cultures by demonstrating that fast-growing cultures infective on lupins carried slow-growing strains which were responsible for the infections.

Infective isolates were numbered serially, and these serial numbers were prefixed with a letter according to the species of the source host (A for *angustifolius*, D for *digitatus* and L for *luteus*). Isolates A10, A11, A13, D24, D25, D26, D27, and L7 were selected for examination. The strain W72, isolated from *L. digitatus* in South Australia (Hockley, priv. comm.), was included as a positive control.

EXPERIMENTAL

Glasshouse experiment

Each strain was assessed for symbiotic ability with each lupin species, in a glasshouse pot experiment of design:

species (3) × treatments (11) × replications (3).

* Details of methods will be provided on request.

Plants were grown in sterilized sand with full nutrients except nitrogen, which was added only in the +N controls. Inoculation was by water suspension of the bacteria placed on the seed. As in the experiments reported by Carroll [3] and Allen and Allen [2], care was taken to control dust, insects and other vehicles permitting contamination of plant containers by rhizobia. The experiment was conducted in a closed glasshouse room ventilated through oiled muslin screens. In addition to preliminary treatments with bactericidal, fungicidal, and insecticidal sprays, the room was sprayed frequently with liquid paraffin emulsified in water with Lissapol N300*. Host legumes were grown in non-drainage glazed pots of sterile nutrient sand, fitted with capped glass watering tubes. When the lupin seedlings were about 6 to 8 cm high the surface of the sand was dressed with sterile oiled vermiculite.

Every fifth pot of each species was not inoculated, and these pots constituted the control series: thus the treatment without nitrogen or *Rhizobium* was designated −N treatment. Half of the uninoculated pots were supplied with 0.2 g of NH_4NO_3 (+N treatment). Pots were randomized within blocks and their water contents maintained at 10% by weight. At 36 days all +N treatments received a further 0.1 g of NH_4NO_3 per pot.

The experiment was terminated at 50 days. Plant tops were harvested, dried, weighed and analysed for total-nitrogen content after the method of Willits, Coe and Ogg [12]. An analysis of variance was performed on the data and the results are summarised in the Range Test Diagrams of Table 1, where any two totals not underlined by the same line are significantly different at the level indicated.

Results. When based on dry-weight data, treatment effects were highly significant, but this was due almost entirely to the single comparison, −N *versus* all other treatments. If the −N treatment was excluded there was no significant difference between remaining treatments. In contrast to the dry-weight data, the total-nitrogen data permitted detection of highly significant differences between treatments.

On *L. luteus*, all inoculation treatments were better than +N treatments at the 1% level of significance. The isolates A10, A11, A13, D24, D25, D26, D27 and W72 were better than L7 at the 1% level. With *L. angustifolius* and *L. digitatus* some differences are detectable at the 5% level of significance.

The host-strain relationship shown in this experiment is dissimilar to that demonstrated in, for example, *Trifolium–Rhizobium* symbiosis, where specific differences in both infectiveness and effectiveness are often marked [9 10 11].

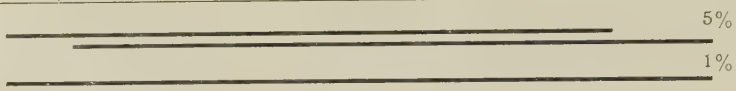
* Product of I.C.I.

TABLE 1

Total nitrogen content in mg of top growth of 3 lupin species on 10 treatments. Glasshouse experiment

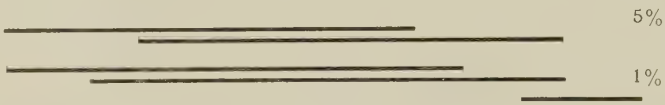
Treatment totals are the sum of 3 estimates of total-nitrogen content per pot (4 plants/pot)

D27	W72	L7	D25	D24	D26	A13	A11	+N	A10	−N
409·9	366·6	325·5	323·5	322·1	315·5	311·3	298·5	296·3	248·7	94·0



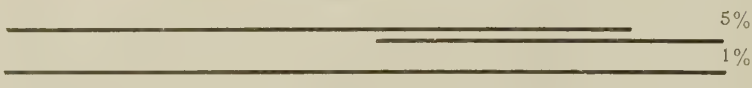
L. angustifolius

D27	D24	A10	A11	D26	W72	D25	A13	L7	+N	−N
376·6	371·3	357·0	354·5	353·3	346·5	329·1	319·0	275·6	223·2	72·0



L. luteus

D24	L7	D25	A10	D26	W72	D27	A13	A11	+N	−N
403·7	395·5	394·3	392·6	386·0	371·7	359·0	348·9	328·3	294·1	120·9



L. digitatus

Any totals not underlined by the same line are significantly different at the level indicated.

Field experiment

Each isolate was assessed for symbiotic ability with each lupin species in a field experiment of factorial design:

species (3) × treatments (12) × replications (3).

The experimental site was located on virgin soil at Bakers Hill, 45 miles east of Perth. An area was subdivided into 108 plots, each 4′ × 4′, separated from one another by paths four feet wide.

All seed was disinfected prior to treatment. Three replicate blocks of each of the three lupin species were planted, giving a total of nine blocks in latin-square design. Each block comprised 12 plots. The nine inoculation treatments were planted each in one plot of each block. The remaining three plots in each block, planted with uninoculated seed, were controls. These were:

−N (no inoculum, no nitrogen)
+N (no inoculum, 600 pounds of NH_4NO_3 per acre)
+2N (no inoculum, 1200 pounds of NH_4NO_3 per acre)

The NH_4NO_3 was applied in three equal dressings, at planting, at six weeks and at the pre-flowering period. A full mineral supplement was added to the soil to allow the maximum expression of symbiotic nitrogen fixation.

Seed was planted in June and plants were harvested in October, at flowering. Plants in each plot were counted, harvested at ground level, bulked, and weighed.

Sub-samples were taken from each plot for the estimation of the dry-weight yield. An analysis of variance was performed on the data and the results are summarized in the Range Test Diagrams of Table 2, where any two totals not underlined by the same line are significantly different at the level indicated. When the plants were 6 weeks old, symptoms of nitrogen stress were obvious in all plants in the control −N plots of all three species. "Stress" plants exhibited orange-coloured cotyledons, often already dehisced, and yellow-tinged primary leaves. These symptoms were also apparent in many inoculated plots and counts were made of them and of the total number of plants. The results of an analysis of variance performed on these data are summarized in the Range Test Diagrams of Table 3.

Results. Responses to inoculation were obvious at harvesting. Nodulation occurred on all uninoculated *L. digitatus* plants but was almost absent in the control plots of the other two species. A similar observation was reported as early as 1930 by Adams and Riches [1], who suggested the possibility of infective specificity in lupins. However, this uncontrolled nodulation did not obscure responses to test inocula. Assessment of this experiment was based on mean dry yields of single plants (Table 2). Figures for −N uninoculated control plants were omitted from the analysis to permit more discrimination between the less grossly divergent strain performances. The mean dry yields of single plants of uninoculated *L. angustifolius* were 0.134 oz.; *L. digitatus* 0.371 oz., and *L. luteus* 0.022 oz.

There were highly significant differences between strain performances. Highest dry-yield figures were obtained with a different bacterial strain on each species, and only D24 was consistent on all three species in producing lowest dry yield. In this context the data of Table 3 are pertinent. At six weeks the D24-treated seedlings had a significantly higher proportion of "stress" plants showing N-starvation, than any other strain treatment ($P < 0.05$), a result which was due to poor, early nodulation.

Spare inoculated seed of all three lupin species from the field experiment was planted in pots of sterilized nutrient sand three weeks after inoculation. Resulting plants mostly failed to nodulate.

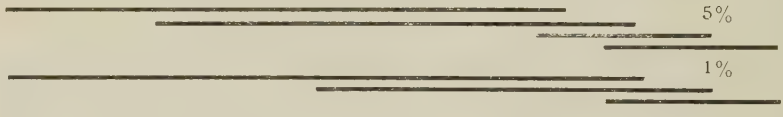
TABLE 2

Estimated dry yields in ounces of 3 lupin species on 11 treatments. Field experiment. Treatment totals are the sum of 3 estimates of dry yield per plant

L7	A13	D25	A11	D27	D26	A10	+2N	W72	D24	+N
2·754	2·623	2·495	2·376	2·217	1·998	1·990	1·610	1·609	1·341	0·962

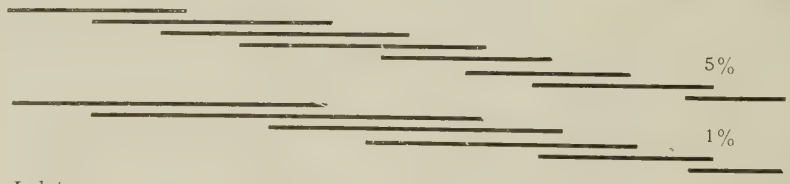
L. angustifolius

D26	A13	A11	D25	W72	D27	L7	A10	+2N	D24	+N
3·816	3·758	3·681	3·618	3·566	3·334	3·329	3·270	2·942	2·516	2·185



L. digitatus

D25	A10	A11	D27	W72	A13	D26	L7	+2N	D24	+N
4·403	3·905	3·794	3·493	3·427	3·039	2·879	2·517	2·228	1·768	1·138



L. luteus

Any totals not underlined by the same line are significantly different at the level indicated.

This suggests that the failure of some inocula to survive on seed planted under certain field conditions could be one factor explaining the differences in strain performances between the glasshouse and field experiments.

TABLE 3

Incidence of nitrogen stress in lupin plants of 3 species on 11 treatments.
Field experiment
To permit analysis of the stress/total plant ratios by the analysis of variance technique, they were transformed using the arc-sin transformation

A11	D25	A13	D27	L7	D26	A10	W72	D24	—N
35·67	38·89	61·10	61·51	62·09	85·57	92·68	112·28	135·13	270·00

5%
1%

L. angustifolius

A11	D27	A10	L7	D25	A13	D26	W72	D24	—N
0·40	8·33	11·97	12·52	12·66	13·44	43·31	64·12	93·36	270·0

5%
1%

L. digitatus

A11	D25	A13	L7	A10	D27	W72	D26	D24	—N
0·24	0·98	10·14	17·05	22·22	41·30	46·66	53·50	151·29	270·0

5%
1%

L. luteus

Any totals not underlined by the same line are significantly different at the level indicated.

Nodulation of lupins by indigenous rhizobia

In an experiment conducted on deep sand in 1957 the control plots were all nodulated to some extent by "indigenous" rhizobia, *L. digitatus* being much more generally nodulated than *L. angustifolius* or *L. luteus* in that order. These plots were therefore harvested separately. The green weights of single plants were recorded, and are presented graphically in the weight frequency diagram of Fig. 1. The differences shown in the weight distributions are highly significant ($P < 0.001$). Similar results have been obtained in three other, widely separated localities.

An examination of Fig. 1 shows conclusively that the species *L. digitatus* was more susceptible to nodulation by soil organisms

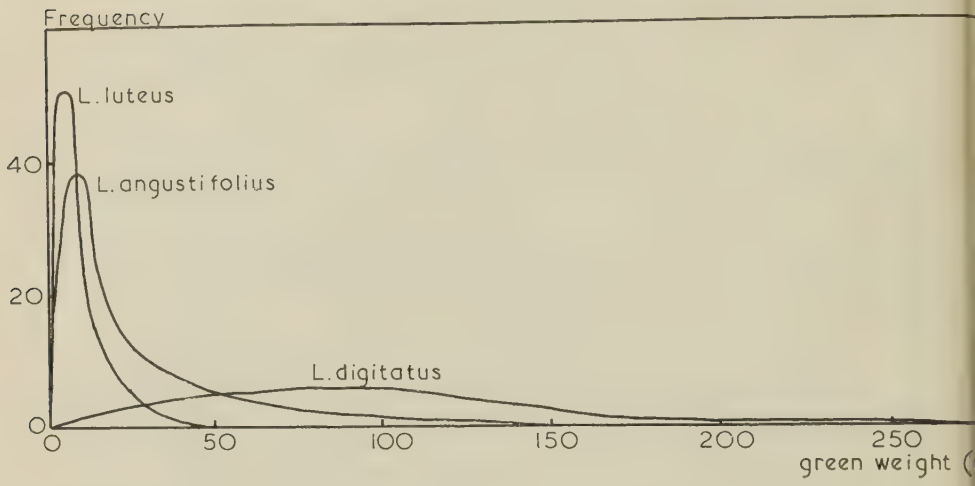


Fig. 1. Weight–frequency distributions of three uninoculated, field-grown lupin species.

than *L. angustifolius* which, in turn, was more susceptible than *L. luteus*. This result is interesting in view of the lack of species specificity shown by the lupin bacteria in our earlier experiments.

DISCUSSION

The nine rhizobial strains isolated from lupins and examined in this study were all effective on *L. angustifolius*, *L. digitatus* and *L. luteus*. The differences between their glasshouse and field performances demonstrate the importance of field conditions in modifying the expression of basic strain capabilities. In the field experiment the differences between treatments with respect to nitrogen stress at six weeks tended to correlate with the differences in the final yields (see Tables 2 and 3). Thus differences in the field performances of strains were as readily ascribable to factors influencing infection of the host by the bacterium as to differences in effectiveness. Harris [8] has suggested incursion as a strain property, independent of effectiveness, but of possible relevance to success in commercial inoculation of clovers.

Given satisfactory field conditions for host infection, strains D25, A10, and A11 have proved the best of the nine strains as

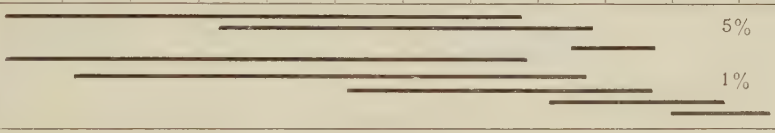
inocula for *L. luteus*. Strains D26, A13, A11, D25, W72, L7, and A10 were equally satisfactory on *L. digitatus* and L7, A13, D25, A11, D27, D26, and A10 were satisfactory inocula for *L. angustifolius*. In the field experiment these listed strains have proved equal to, and on two species superior to W72, the strain currently used for lupin inoculation in Australia.

The most versatile of the strains is D25, as can be seen from a study of Table 4, which is derived from the data presented in Table 2. It is also noteworthy that the best strains produced significantly higher yields than did ammonium nitrate at 1200

TABLE 4

Summated dry yields in ounces of 3 lupin species on 11 treatments.
Field experiment
Treatment totals are the sum of 3 estimates/species of dry yield per plant

D25	A11	A13	A10	D27	D26	W72	L7	+2N	D24	+N	−N
10·516	9·851	9·420	9·165	9·044	8·693	8·602	8·600	6·780	5·625	4·285	1·572



Any totals not underlined by the same line are significantly different at the level indicated.

pounds per acre in the field and at approximately 600 pounds per acre in pots.

No association between strain source and strain performance was detected in either the glasshouse or the field experiment.

The appearance of species differences with respect to the nodulation of lupins in the field by indigenous rhizobia is difficult to explain, considering the complete lack of any such differences in controlled experiments with lupin bacteria. However, the former result has been supported by experiments in this laboratory with pure-culture isolates from native legumes (unpublished data) in which *L. digitatus* was found more susceptible to infection than either *L. angustifolius* or *L. luteus*. An intriguing difference has therefore been established between the symbiotic behaviour of lupin bacteria and those isolated from native legumes. Further work is in progress on the relationships between native rhizobia and introduced legumes.

SUMMARY

Bacteria from the nodules of *Lupinus angustifolius* L., *L. digitatus* Forsk., and *L. luteus* L. have been isolated, and the symbiotic relationships of nine of these strains and the three lupin species investigated in a glasshouse experiment. All symbioses were effective and no interaction or specificity was detected among the symbionts.

Soil factors modified expression of the strains' symbiotic abilities in the field, but several strains have shown successful symbiosis with all three lupin species.

One lupin species, *L. digitatus*, nodulates freely under natural conditions in Western Australia, whereas *L. luteus* and *L. angustifolius* do not. This lupin is more readily infected by native strains of rhizobia than the others.

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INFLUENCE ON PLANT GROWTH OF THE BREAKDOWN OF ORGANIC PHOSPHORUS COMPOUNDS BY MICRO-ORGANISMS

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INTRODUCTION

Although numerous investigations have been made on phosphorus-containing organic compounds in the soil and their uptake by higher plants, the subject is not as yet fully understood. Various workers have produced numerous but often conflicting results. Merely to determine the content of organic phosphorus and its many constituents is itself a problem, and it is therefore not surprising that so little is known.

It is generally accepted that up to 50 per cent of the total P in the soil may be in the organic form; depending on the soil, some 5 to 50 per cent of this has been isolated as certain defined fractions (Kaila [14]). The largest fraction of the organic P — approximately 50 per cent — appears to be in the form of phytin and its derivatives (Bower [2], Pederson [19]). The nucleic-acid content of the soil is not known; certain authors would suggest that it comprises only about 5 per cent of the organic P (Dyer and Wrenshall [4], Adams *et al.* [1]). The amount of lecithin in the soil is small, amounting only to about 1 per cent of the total soil P (Bower [2]). The remainder of the organic soil P occurs in as yet unknown forms (Kaila [14]).

The exchange of P in the soil is so rapid that it has been very difficult to follow its course (Goring [8 9]); there is no doubt however, that in this, soil organisms play a significant role (Feher [5], Kaila [12 14], Mienkina [17], Kudrin [16], Gołębiowska and Myśków [10], Kobus [15], Szember [24]).

Certain authors state that plants, or at least certain species of

plants, can utilize organic P without the aid of micro-organisms, either directly or by first hydrolyzing the compound by means of enzymic systems present in the roots. According to Rogers *et al.* [20] and Weissflog and Mengdehl [26], peas and corn can utilize phytin P, whilst lecithin P can be utilized by corn and tomato. The evidence for phytin uptake by higher plants is further supported by the work of Bower [2], Heck and Whiting [11] and others. Readers particularly interested in this subject are referred to the review by Ulrich and Benzler [25].

Whether plants are or are not capable of utilizing P from its organic compounds, it is almost certain that the presence of micro-organisms in the soil may increase the amount of readily available P (Feher [5], Kaila [13]). At the same time it must be remembered that in certain instances, micro-organisms may make P unavailable to plants, resulting in P-deficiency in the soil; this often happens when readily available sources of energy are present for the micro-organisms (Kaila [13]).

So as to obtain further information about the direct uptake by higher plants of P from organic compounds, without the aid of micro-organisms, and also about the interrelations between higher plants and micro-organisms, a number of workers have experimented with higher plants grown under sterile conditions (Weissflog and Mengdehl [26], Gerretsen [7], Sobieszczański [22], Nilsson [18], and others).

In order to elucidate these phenomena more completely, the aims of the present investigation have been to isolate from the natural environment, organisms active in the breakdown of phytin and lecithin and to test these organisms by inoculation of cultures of plants grown under sterile conditions.

MATERIALS AND METHODS

The micro-organisms capable of breaking down phytin and lecithin were isolated by an enrichment technique. The basic liquid enrichment medium contained (in tap water):

NaCl	0.01%	$MnSO_4.7H_2O$	trace
$MgSO_4.7H_2O$	0.02%	K_2SO_4	0.03%
$FeSO_4.7H_2O$	trace	$CaSO_4$	0.01%

To this was added either lecithin (from N.V. Amsterdamsche Chininefabriek) at 0.05%, or phytin (calcium magnesium inosito-hexaphosphoricum,

from T. Schuchard) at 0.2%. In some experiments 0.05% NH_4NO_3 and/or 1.0% glucose were also added.

The various prepared media were left in open, unsterilized dishes for about 4 weeks, inoculated with soil. At intervals, samples were removed and plated onto similar media in agar. Colonies which produced a clearing in the agar medium were isolated for further investigation.

The influence of the addition to the medium of N, P, and sugar, on the breakdown of lecithin or phytin by the isolated organisms was also investigated. So as to determine the amount of P liberated from the lecithin or phytin, the organisms were grown on the above-mentioned liquid medium. The breakdown of lecithin was studied under both aerobic and partially anaerobic conditions (in long tubes), at temperatures of 20° and 32°C. Similarly, the breakdown of phytin was studied under aerobic conditions (rotary shaker) and under anaerobic conditions, using Hall-Barker tubes. The capacity of these organisms to break down phytin in the absence of sugar in the medium was also investigated.

The amount of P liberated was measured by the Scheel modification [21] of the Fiske and Subbarrow method, using the Beckman DU spectrophotometer. All experiments were made in triplicate. Since lecithin produced an opalescent medium which interfered with the colorimetric determinations, the phosphate was removed from the medium by ion-exchange resin (Anionenaustauscher IRA 400). The amount of phosphate was then determined colorimetrically in the resulting eluate. In the phytin experiments, simple centrifugation removed all sources of interference in the colorimetric analysis.

For examining the suitability of the isolated strains of organisms as inocula for higher plants, aseptically grown radish plants were used. The plants were grown in glass cylinders, 5 cm in diameter and approximately 30 cm long, plugged with cotton wool. To provide sterile growth conditions, the cylinders were autoclaved with their corresponding contents; in the case of quartz-sand substrates, sterilization was carried out at 110°C for 1 hour. The seeds were sterilized in the apparatus suggested by Gerretsen [6] and for germination, were transferred aseptically to petri dishes containing a sterile agar medium which readily revealed normal contamination. Those germinated seeds which showed no signs of contamination were transferred aseptically to the glass cylinders.

Three series of experiments were set up: in the first, the substrate consisted of quartz sand mixed with the appropriate nutrients, in the second, also sterile, of agar with the appropriate nutrients added; the third series was made under non-sterile conditions, using the quartz-sand substrate.

All treatments within each series were replicated 10 times; the treatments were:

1. Knop's medium without phosphate.
2. ,, ,, with phosphate.
3. ,, ,, with lecithin instead of phosphate.

4. Knop's medium with lecithin instead of phosphate and inoculated with one of the isolated micro–organisms.
5. ,, ,, with phytin instead of phosphate.
6. ,, ,, with phytin instead of phosphate, and inoculated with one of the isolated micro-organisms.

At the end of the growth period, the fresh and dry weights of the plants were recorded and determinations made of the uptake of N and P. The sterility of the vessels not deliberately inoculated was checked at the conclusion of the experiments.

RESULTS

Isolation and characteristics of the micro-organisms

Organisms which utilized lecithin as a source of P, N, and C, and which produced clear areas in the lecithin-agar medium, could readily be isolated. Colonies of two such strains are illustrated in Plate 1A and B.

The organism shown in Plate 1A was a small rod, that in Plate 1B, an actinomycete: in the following tables these are recorded as A7 and P8 respectively. In all, twenty lecithin-decomposing organisms were isolated; most of these were very short rods but one actinomycete was found.

The effect of the addition to the agar medium of a source of nitrogen, phosphate, or glucose on growth and on the breakdown of lecithin is recorded in Table 1.

Judging by the action of the organisms producing clear areas around their colonies, the addition of N to a lecithin-containing medium does not increase the breakdown of lecithin by these organisms. In certain instances in fact, it was possible to observe a diminution or even an inhibition of this action (Table 1, strains A1, A2, A3, C2). The addition of phosphate, in all cases except that with strain P8 – the actinomycete – entirely suppressed the breakdown of lecithin. In general, the addition of glucose to the medium also inhibited or retarded the breakdown of lecithin by the organisms tested.

The five most active strains were chosen and the amount of phosphate liberated by them from lecithin in the above liquid medium was determined; the results are given in Table 2.

Apparently considerable amounts of lecithin are decomposed within the short incubation period. The reaction of the various

TABLE 1

Influence of the addition to an agar medium of NH_4NO_3, KH_2PO_4, or glucose on the breakdown of lecithin by various organisms.
Legend: G = growth, B = breakdown, + = weak, ++ = strong, +++ = very strong, ○ = none

Strain No.	Additions to medium: 0		NH_4NO_3		K_2HPO_4		NH_4NO_3 K_2HPO_4		NH_4NO_3 glucose		Remarks
	G	B	G	B	G	B	G	B	G	B	
A1	++	++	++	+	++	○	+++	○	+++	○	
A2	+	+	+	○	+	○	++	○	+++	○	
A3	+	+	+	○	+	○	+	○	+	○	
A4	+	+	+	+	+	○	+	○	+	○	Breaks down phytin also
A6	+	+	+	+	+	○	+	○	+	○	
A7	+	++	+	++	+	○	+	○	+++	○	
A8	++	++	++	++	++	○	+++	○	+++	+	
A9	++	++	++	++	++	○	+++	○	+++	+	
A10	++	++	++	++	++	○	+	○	+++	+	
A11	++	++	++	++	++	○	+	○	+++	○	
A21	+	++	+	++	+	○	++	○	+	+	
A22	+	++	+	++	+	○	+	○	+	+	Breaks down phytin also
B1	+	+	+	+	+	○	+	○	++	+	
B5	+	+++	+	+++	+	○	+	○	+	+	
B6	++	++	++	++	+	○	++	○	++	○	
C1	+	+	+	+	++	○	+++	○	+++	○	Breaks down phytin also
C2	+	+	+	+	+	○	+	○	+	○	,,
C3	+	+	+	+	+	○	+	○	+	+	,,
C4	+	+	+	+	+	○	+	○	+	+	
P8	+	+	+	+	+	○	+	○	+	○	

TABLE 2

Amount of phosphate (mg P_2O_5/100 ml) liberated from lecithin after 7 days incubation at two different temperatures

Strain no.	Aerobic		Anaerobic	
	20°C	32°C	20°C	32°C
A10	3.6	2.0	2.3	2.5
A22	2.1	1.7	1.1	1.6
B5	1.9	2.3	4.6	4.0
C3	2.3	3.1	2.4	2.8
P8	4.7	2.9	3.5	4.9

strains examined to changes in temperature and aeration was rather variable.

Twenty-seven strains, including four yeasts and one actinomycete were isolated which produced clear areas around their colonies on the phytin-agar medium. None of the colonies of any strain examined – with the exception of the actinomycete – produced these halos on the agar medium in the absence of glucose.

The colonies of the strains F_1, F_2, F_3, F_4, F_{15} and F_{22} are shown in Plates 1C–F and 2A and B, while stained cultures are illustrated in Plate 2C and D.

These organisms belong to various morphological groups. Besides short rods and cocci, very long, sometimes spore-forming rods (strain F1) were found. One of the strains was motile (F22); some liquified gelatine (F20). These general observations suggest that the organisms so isolated do not belong to one systematic group specialized for the breakdown of P-containing organic compounds: on the contrary, this property appears to be spread over many taxonomic units.

The decomposition of phytin in liquid medium by the most active strains is recorded in Table 3.

TABLE 3

Amount of phosphate (mg P_2O_5/100 ml medium) liberated from phytin in liquid medium					
Strain no.	Aerobic		Anaerobic		Remarks
	Medium with glucose	Medium without glucose	Medium with glucose	Medium without glucose	
F15	4.1	1.7	2.8	1.4	After 14 days incubation
F20	3.8	1.2	0.0	0.0	„
F21	2.9	0.2	0.6	0.0	„
F27	0.0	1.9	0.0	0.0	After 7 days incubation

It will be seen from Table 3 that all the strains examined were more active under aerobic conditions. The first three strains in the table (bacteria) broke down phytin more rapidly in the presence of glucose; the last strain (actinomycete), which produced only a slightly clear area around its colony in the phytin-agar medium liberated phosphate readily in the absence of glucose. Addition of glucose to the medium led to rapid growth of the actinomycete,

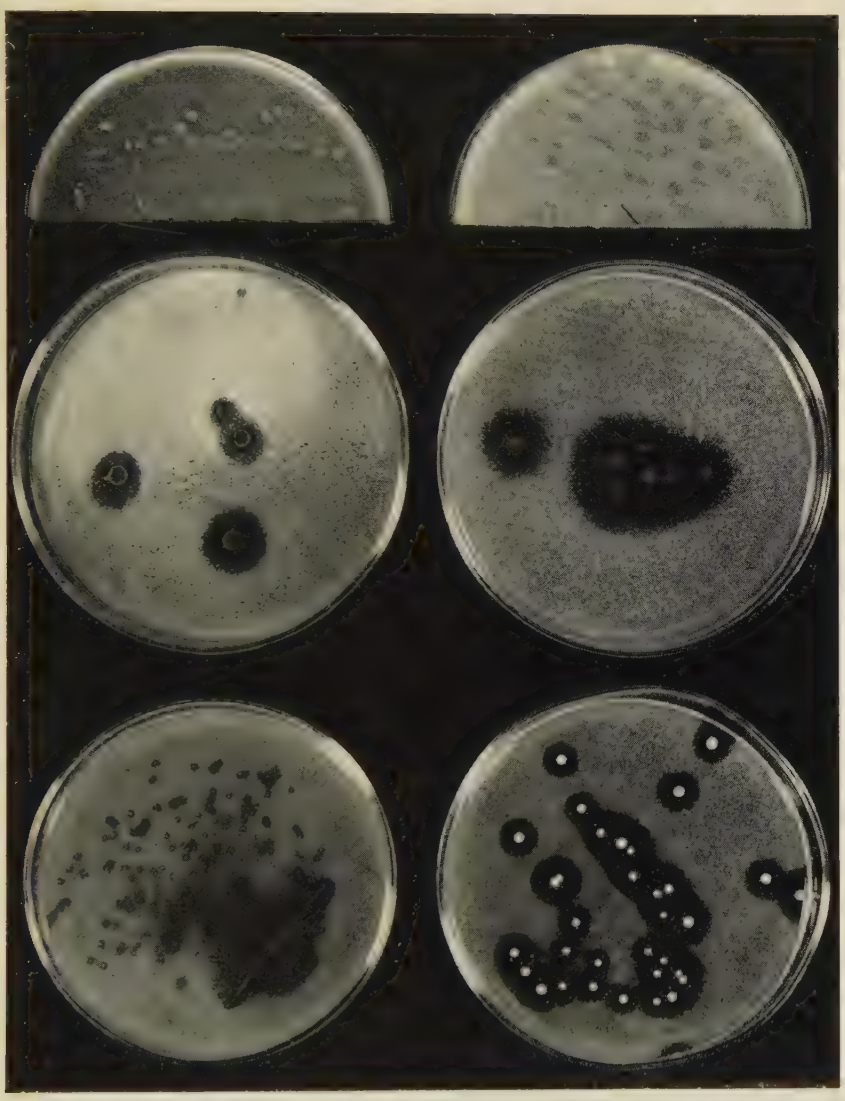

Plate 1. **A** and **B**:- Lecithin-decomposing organisms on lecithin agar. A - small rod, recorded as A7; B - actinomycete, recorded as P8. **C** to **F**:- Colonies of phytin-decomposing organisms, surrounded by clear areas, on phytin agar. C - F1; D - F2; E - F3; F - F4. (Magnification $^1/_2\times$)

Plate 2. **A** and **B**:- Colonies of phytin-decomposing organisms surrounded by clear areas, on phytin agar. (Magn. 3×). A - F15; B - F22. **C** and **D**:- Rods of F15 (C) and F22 (D) colonies stained with carbol fuchsin (Magn. 1500×)

but under the conditions of the experiment, no phosphate could then be detected in the medium. Since it is known that phytin contains a certain amount of free phosphate, this is apparently momentarily utilized by the actinomycete (F27) following the addition of glucose. It may be assumed that this organism breaks down phytin only when sources of energy other than phytin are absent; apparently under such conditions more P is produced than is necessary for the metabolism of the organism, hence its liberation into the medium.

Effect on phosphorus nutrition of plants

In order to answer the question as to how these organisms affect the P-nutrition of plants, the two most active strains – B5, a lecithin and F16, a phytin decomposer – were used to inoculate cultures of plants grown under sterile conditions. Figure 1 illustrates such cultures of radish plants grown on a sand substrate; Figure 2 illustrates a similar series grown on agar. In both these cultures, the plants were raised in glass cylinders plugged with cotton wool. In a third series – the controls – the plants were grown on sand under non-sterile conditions (Figure 3). The numbers on the photographs correspond to the particular treatments listed under 'Materials and methods'.

Fig. 1. Radish plants grown on sterile sand with Knop's solution.
Treatments: 1 – without phosphate; 2 – with phosphate; 3 – without phosphate, with lecithin; 4 – as 3 but inoculated with a lecithin-decomposing organism, B5; 5 – without phosphate, with phytin; 6 – as 5 but inoculated with a phytin-decomposing organism, F16.

Fig. 2. Radish plants grown on sterile agar with Knop's solution. Treatments as in Fig. 1.

Fig. 3. Radish plants grown on *non*-sterile sand with Knop's solution. Treatments as in Fig. 1.

In all three series, growth on the P-deficient substrate 1 (Knop minus P) was much less than that on the other substrates, between which significant differences scarcely could be observed visually.

When sufficient growth had been made, the plants were harvested and weighed. The results are recorded in Table 4; Table 5 gives the N- and P-contents on a dry-weight basis *.

As will be seen from Table 4, both lecithin and phytin can serve as a source of P for plants. The best growth was apparently obtained on media containing phytin (5 and 6) and the average weight

* The author wishes to express his thanks to Miss R. van der Mei for the N- and P-analysis of the dried plant material.

TABLE 4

Fresh weights (g) of plants grown on the different media

Treatment *	Medium		
	Sterile, agar	Sterile, sand	Non-sterile, sand
1	0.8	1.0	0.8
2	2.4	2.9	3.5
3	1.8	1.4	2.5
4	1.8	1.5	2.2
5	2.4	2.3	3.4
6	2.5	2.9	4.4

* See 'Materials and methods'.

TABLE 5

N- and P-contents (percent of dry matter) of plants grown on the different media

Treatment *	Medium					
	Sterile, sand		Sterile, agar		Non-sterile, sand	
	N	P	N	P	N	P
1	2.5	0.22	5.4	0.33	2.6	0.16
2	3.8	0.74	5.6	1.8	2.4	0.52
3	2.6	0.37	5.1	1.8	3.0	0.22
4	2.4	0.41	4.8	1.1	2.9	0.25
5	3.6	0.52	5.6	2.4	2.5	1.33
6	3.2	0.47	6.5	2.7	2.5	1.33

* See 'Materials and methods'.

of these plants equals or, when inoculated, may even surpass the weight of the plants grown on the complete Knop medium (2). Inoculation with strains which break down lecithin did not appear to increase the growth of the plants. According to Table 5, it is evident that not only the growth, but even more so the P-uptake was much greater with phytin than with lecithin, especially under non-sterile conditions.

Generally the best growth was obtained with plants grown under non-sterile conditions: this would be expected since in the sterile cultures, growth conditions were rather unnatural. It is interesting to note that the plants grown under non-sterile conditions on the lecithin media (3 and 4) took up P to a lower percentage than those grown under sterile conditions. Plants grown under sterile conditions on the phytin-containing medium produced clear areas around the roots. This confirms the suggestion that the plant is able to break down phytin without the aid of micro-organisms.

Prior to harvesting the sterility of the aseptic cultures was checked and only in a few cases was there evidence of slight contamination; because of the large number of replicates – ten – the contaminated cultures could be excluded in the calculation of average values.

It was tried to repeat this experiment, however only with phytin and with an other plant – kale – cultivating it in a way as to allow for the maintenance of complete sterility of soil and roots and at the same time providing the tops with as near as possible natural conditions. Such a culture apparatus is now being used by the author and is based on the suggestions of Gerretsen [6] with modifications for the supply of water through a bacterial filter (Szember [23]). This set-up, however, has not given better results: again was the growth on the phytin-containing sand medium fairly good, but worse than on the complete Knop solution, while the inoculation of the phytin medium with strain F15 proved to have an adverse effect, presumably by a toxicity of the microbe used. Fig. 4 illustrates this experiment.

Fig. 4. Kale plants grown in sterile sand with Knop's solution. *Treatments:* 1 – without phosphate, with phytin; 2 – as 1 but inoculated with strain F15; 3 – with phosphate; 4 – without phosphate. The dry-matter yields of these plants were: 1 – 2.01; 2 – 1.00; 3 – 2.65; and 4 – 0.25 grams.

CONCLUSIONS

It has been confirmed that micro-organisms capable of breaking down phytin or lecithin can readily be isolated from soil. Until now however, the relative activity of the various strains has not been studied in detail and it was considered necessary therefore, to

investigate the phenomenon under conditions approaching as closely as possible those applying in the soil.

Under the conditions of the present experiments, the inoculation of cultures of higher plants with organisms capable of breaking down phytin gave sometimes an only slightly positive result and sometimes affected the plant adversely, while the inoculation of the plants grown on the lecithin-containing medium had no significant effect whatsoever (Tables 4 and 5). It must be noted however, that these experiments were made with only one strain for each substrate and with only two species of plants; to obtain a more general insight therefore, it would be necessary to extend the investigations to include other strains and other plants.

SUMMARY

Micro-organisms which break down lecithin or phytin have been isolated from soil by enrichment cultures. Only those organisms were investigated which on an agar medium containing lecithin or phytin as the sole source of P (in the case of lecithin, also as a source of N and energy), produced a clear area around their colonies. Certain of these organisms were inoculated into sterile cultures of radish plants grown on sand or agar substrates containing lecithin or phytin as the source of P, and the growth and N- and P-uptakes of the plants were compared with those of similar plants grown in non-sterile sand cultures or supplied with KH_2PO_4. Evidence was obtained that both lecithin and phytin can serve as P-sources for higher plants even under sterile conditions, phytin producing a greater effect than lecithin. Inoculation, however, had only in the phytin-containing medium sometimes a slight effect on the P-nutrition of the plants and further work in this field is therefore necessary.

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ZUR GEGENSEITIGEN UMWANDLUNG VON CALCIUMGEBUNDENER PHOSPHORSÄURE UND ALUMINIUM- UND EISEN (3)-GEBUNDENER PHOSPHORSÄURE IM BODEN

von WERNER RATHJE *

EINLEITUNG

Früher [5] wurde nachgewiesen, dass isomorpher Hydroxylfluorapatit mit einem Gehalt von 30% Hydroxylapatit und 70% Fluorapatit bei Gegenwart von Calciumfluorid existenzfähig ist, und das am schwersten lösliche Calciumphosphat ist. Da im Boden Calciumfluorid vorhanden ist [9 10], stellt also der Hydroxylfluorapatit das Calciumphosphat-Mineral des Bodens [12] und der Rohphosphate [5] dar. Als stöchiometrisch definierte Verbindung besitzt er ein bestimmtes Löslichkeitsgleichgewicht, in welchem etwa 0,1 mg P_2O_5 je Liter enthalten ist. Bei saurer Reaktion ist dieses Löslichkeitsgleichgewicht höher als bei alkalischer Reaktion, weil der Hydroxylfluorapatit ein Salz einer starken Base und einer schwachen Säure ist.

Weiterhin wurde gezeigt, dass reines Aluminiumphosphat, $AlPO_4.2H_2O$ und reines Eisen(3)phosphat, $FePO_4.2{,}5\,H_2O$ nur bei so hohen Phosphat- und Wasserstoffionenkonzentrationen existenzfähig sind [7 8], wie sie im Boden nicht vorkommen, und die aluminium- und eisen (3)-gebundene Phosphorsäure des Bodens daher in Form der Austauschersubstanzen Aluminiumhydroxydphosphat und Eisen(3)-hydroxydphosphat vorhanden ist [13]. Da die Austauschersubstanzen allgemein das Kennzeichen besitzen, dass in ihnen die Molarprozente der austauschbaren Ionen in weitem Bereich eine Funktion der Konzentrationen dieser Ionen im Gleichgewicht darstellen [6], sind die Austauschgleichgewichte der Aluminium- und Eisen(3)-hydroxydphosphate von der Menge der adsorbierten Phosphat- und Hydroxylionen abhängig. Daher sind die im Gleichgewicht vorhandenen Phosphatkonzentrationen von extrem kleinen [14] bis zu sehr hohen Werten [7 8] veränderlich und bei alkalischer Reaktion höher als bei saurer Reaktion.

Im Boden enthalten die Aluminium- und Eisen(3)hydroxydphosphate daher so hohe Molarprozente an adsorbierten Phosphationen, dass in ihren Austauschgleichgewichten bei jedem pH-Wert

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ebenso hohe Phosphatkonzentrationen enthalten sind [12] wie im Löslichkeitsgleichgewicht des Hydroxylfluorapatits (Kurve 1) *. Es müsste daher möglich sein, im Boden durch Erhöhung (Erniedrigung) der Wasserstoffionenkonzentration die Menge der an Aluminium- und Eisen(3)-hydroxydphosphat gebundenen Phosphationen zu vermindern (erhöhen), und gleichzeitig die Menge des Hydroxylfluorapatits zu vergrössern (verkleinern). Diese Möglichkeit ist bereits öfter diskutiert worden [1 2 3 4 11 15 16] und soll im Folgenden durch Umsetzungen an den reinen Phosphatmineralien des Bodens ohne Anwendung von Extraktionsmitteln untersucht werden.

VERSUCHE

1. *Überführung von calciumgebundener Phosphorsäure in aluminium- und eisen(3)-gebundene Phosphorsäure*

a. Qualitative Umsetzung. In mehreren Ansätzen wurden, wie früher beschrieben [5], wässrige Suspensionen von je 0,1 Millimol = 100,6 mg isomorpher Hydroxylfluorapatit hergestellt, in welchem also je 1 Millimol CaO und 0,3 Millimol P_2O_5 enthalten sind. Dazu wurden Suspensionen von je 6 Millimol frisch gefälltem Aluminium- oder Eisen(3)-hydroxyd gegeben (= 3 Millimol Al_2O_3 oder Fe_2O_3). Nach Auffüllen auf 1 l wurde etwas Bromthymolblau und wenig Salzsäure bis zur Einstellung der sauren Färbung des Indikators zugefügt. Zur Erhöhung der Umsetzungsgeschwindigkeit wurde zum Sieden erhitzt. Hierbei wurde die Entstehung von Hydroxylionen (Umschlag zur alkalischen Indikatorfarbe) beobachtet, und im Filtrat die Anwesenheit von gelösten Calciumionen nachgewiesen. Es dürfte daher folgende Reaktionen stattgefunden haben:

$$\text{Calciumphosphat (Hydroxylfluorapatit)} + \text{Aluminium-/Eisen(3)-Hydroxyd} \rightleftharpoons$$

$$\rightleftharpoons \text{Aluminium-/Eisen(3)-Hydroxydphosphat} + \text{Calciumhydroxyd} \quad (1)$$

Calciumgebundene Phosphorsäure wurde also z.T. in aluminium- und eisen(3)-gebundene Phosphorsäuren übergeführt.

* Über Umsetzungen zwischen Ionenaustauschern und schwerlöslichen Salzen s. Griessbach [6], S. 77.

b. Einstellung von Gleichgewichten. Diese Umsetzung lässt sich durch Zugabe von Säure von links nach rechts verschieben. Durch Messung der pH-Werte und der Calciumkonzentrationen kann bestimmt werden, wie weit nach rechts diese Umsetzung verlaufen ist.

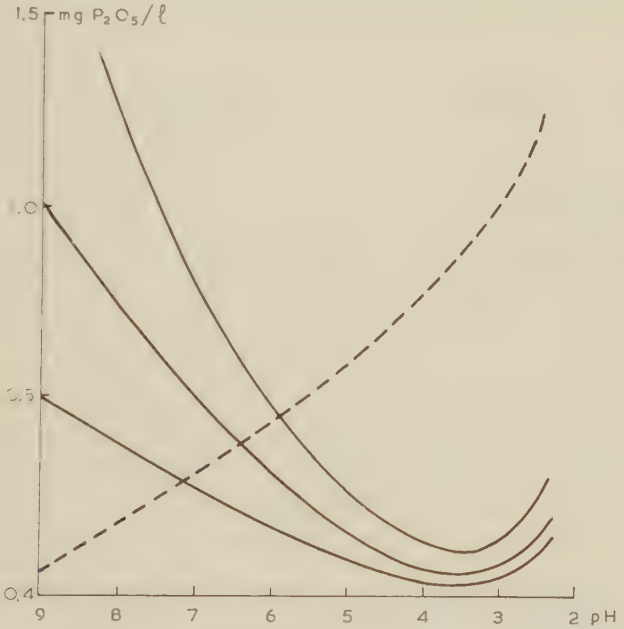

Abb. 1. Löslichkeitsgleichgewichte von Hydroxylfluorapatit und von Aluminium- und Eisen(3)Hydroxydphosphat mit verschiedenen Molarprozenten Phosphat bei verschiedenen pH-Wert.

– – – – – – – – Hydroxylfluorapatit
———————— Aluminium- und Eisen(3)-Hydroxydphosphat.

Nach Zugabe von steigenden Mengen N 10 Salzsäure zu den oben beschriebenen Ansätzen (ohne Indikatorzusatz) wurden die Suspensionen unter Ersatz des verdampfenden Wassers 30 Minuten * zum Sieden erhitzt. Anschliessend wurden Proben aus den Suspensionen entnommen, in denen nach Abkühlen der pH-Wert (Glas-

* Es wurde gefunden, dass nach etwa 10 bis 15 Minuten langem Erhitzen zum Sieden keine Konzentrationsveränderungen mehr auftraten, also die Einstellung von Gleichgewichten erreicht sein dürfte.

elektrode) und nach sofortigem Filtrieren die Konzentrationen an gelöstem Calcium [18] und Phosphat [17] bestimmt wurden (Tab. 1).

TABELLE 1

Calcium- und Phosphatkonzentrationen in Suspensionen von Hydroxylfluorapatit und Aluminium- und Eisen(3)-Hydroxyd im Gleichgewicht bei verschiedenem pH-Wert

Zugesetzte ccm $N/10$ HCl	pH	In Lösung befindliche		Im Gleichgewicht vorhandene		
		mg CaO/l	mg P_2O_5/l	mMol † Hydroxyl-fluorapatit	mMol P_2O_5 als Hydro-xylfluorapa-tit (Ca-gebun-dene Phos-phorsäure)	mMol*P_2O_5 adsorbiert an Al- oder Fe-Hydroxyd
a. mit Aluminiumhydroxyd						
0	9.0	5	0,1	0,09	0,27	0,03
2	8,2	10	0,3	0,08	0,24	0,06
4	8,0	20	0,3	0,06	0,18	0,12
8	6.9	35	0,2	0,04	0,12	0,18
16	5,3	50	0,3	0,01	0,03	0,27
b. mit Eisen(3)hydroxyd						
0	9,0	5	0,1	0,09	0,27	0,03
2	8,8	7	0,1	0,08	0,25	0,05
4	8,5	16	0,1	0,05	0,20	0,10
8	6,4	35	0,2	0,03	0,10	0,20
16	6,0	44	0,2	0,02	0,05	0,25

† Errechnet aus der Differenz angewandte Menge (0,1 mMol) Hydroxylfluorapatit — gelöste Men Calcium.

* Errechnet aus der Differenz angewandte Menge Hydroxylfluorapatit (enthaltend 0,3 mMol P_2O — Menge Hydroxylfluorapatit im Gleichgewicht.

Aus der Tabelle ist ersichtlich, dass im Gleichgewicht mit steigender Wasserstoffionenkonzentration die Menge Hydroxylfluorapatit abnimmt und die Menge des an Aluminium- und Eisen(3)-Hydroxyd adsorbierten Phosphates ansteigt.

2. *Umwandlung von aluminium- und eisen(3)-gebundener Phosphorsäure in calciumgebundene Phosphorsäure*

a. Qualitative Umsetzung. Wie früher [13] beschrieben, wurden Suspensionen von Aluminium- oder Eisen(3)-Hydroxydphosphat hergestellt, die je 6 Millimol Aluminium oder Eisen (= 3 Millimol Al_2O_3 oder Fe_2O_3) und 0,6 Millimol Phosphat (= 3 Millimol P_2O_5) sowie 0,2 Millimol Fluorid enthielten.

Diese Suspensionen in je 1 l Wasser wurden mit Bromthymolblau und einigen Tropfen gesättigter Calciumhydroxydlösung (0,021 *M*)

bis zur Blaufärbung versetzt und unter Ersatz des verdampfenden Wassers zum Sieden erhitzt. Nach kurzer Zeit schlug die Indikatorfarbe von Blau auf Grün um (Verminderung der Hydroxylionenkonzentration). Nach dem Filtrieren liessen sich im Filtrat keine gelösten Calciumionen mehr mit Oxalat nachweisen. Daher dürfte eine Umsetzung nach Gl. (2) erfolgt sein, welche die Umkehrung der Gleichung (1) darstellt:

$$\text{Aluminium-}\atop\text{Eisen (3)-}\text{Hydroxydphosphat} + \text{Calciumhydroxyd} \rightleftharpoons$$

$$\rightleftharpoons \text{Aluminium-}\atop\text{Eisen (3)-}\text{Hydroxyd} + \text{Calciumphosphat}\atop\text{(Hydroxylfluorapatit)} \qquad (2)$$

Die aluminium- und eisen-gebundene Phosphorsäure wurde also z.T. in calciumgebundene Phosphorsäure übergeführt.

TABELLE 2

Wert, Calcium- und Phosphatkonzentrationen in Suspensionen von Aluminium- und Eisen(3)-Hydroxydphosphat nach steigenden Zusätzen Calciumhydroxyd im Gleichgewicht

Zugesetzte				In Lösung befindliche		Im Gleichgewicht vorhandene		
n 1 *M* $H)_2$– mg	mMol = mg CaO	= mg CaO	pH	mg CaO/l	mg P_2O_5/l	mMol Hydroxylfluorapatit †	mMol P_2O_5 als Hydroxylfluorapat. (Ca-geb. P_2O_5)	mMol P_2O_5 an Aluminium od. Eisen(3)-Hydroxyd adsorbiert *
miniumhydroxydphosphatx								
	0	0	5,0	0	0,1	0	0	0
,7	0,1	5,6	5,3	0	0,1	0,01	0,03	0,27
,5	0,2	11,2	6,9	1,5	0,1	0,02	0,06	0,24
,0	0,4	22,4	8,0	3	0,1	0,03	0,09	0,21
,1	0,8	44,8	8,7	11	0,1	0,06	0,18	0,21
,2	1,6	89,6	9,2	40	0,05	0,09	0,27	0,03
en(3)hydroxydphosphat								
	0	0	5,6	0	0,1	0	0	0,3
,7	0,1	5,6	6,0	0,1	0,1	0,01	0,03	0,27
,5	0,2	11,2	7,5	2	0,1	0,02	0,06	0,24
,0	0,4	22,4	8,2	8	0,2	0,03	0,09	0,21
,1	0,8	44,8	9,0	25	0,05	0,04	0,12	0,18
,2	1,6	89,6	9,4	40	0,05	0,09	0,27	0,03

echnet aus der Differenz zugesetzte — gelöste Menge Calcium.
echnet aus der Differenz angewandte Menge P_2O_5 — P_2O_5 als Hydroxylapatit.

b. Einstellung von Gleichgewichten. Durch weiteren Zusatz von Calciumhydroxyd lässt sich diese Reaktion von links nach rechts verschieben, und aus der Höhe des Zusatzes sowie durch Messung der pH-Werte und der Calciumkonzentrationen kann bestimmt werden, wie weit nach rechts diese Umsetzung bei Zusatz verschiedener Calciumhydroxydmengen verlaufen ist.

Zu den Suspensionen ohne Indikatorzusatz wurde also Calciumhydroxyd in steigenden Mengen zugesetzt und unter Ersatz des verdampfenden Wassers 30 Minuten * zum Sieden erhitzt. Anschliessend wurden Proben aus den Suspensionen entnommen, in denen nach Abkühlen der pH-Wert (Glaselektrode) und nach sofortigem Filtrieren die Konzentrationen an gelöstem Calcium und Phosphat bestimmt wurden (Tab. 2).

Aus der Tabelle geht die gleiche Erscheinung wie aus Tabelle 1 hervor, ein Hinweis dafür, dass die Einstellung des Gleichgewichtes von der einen *und* von der anderen Seite der Gl. (1) und (2) erfolgen kann. Quantitativ betrachtet, sind in Tabelle 1 bei gleichem pH-Wert grössere Mengen Hydroxylfluorapatit als in Tabelle 2 vorhanden, ein Befund, der mit der Anwesenheit von gelöstem Calciumchlorid in den Versuchen der Tabelle 1 erklärt werden kann.

ZUSAMMENFASSUNG

Es wurde experimentell gefunden, dass die synthetischen Phosphatmineralien des Bodens (Hydroxylfluorapatit, Aluminium- und Eisen(3)-Hydroxydphosphat) miteinander in einem Gleichgewicht stehen, welches von beiden Seiten der Gleichung eingestellt wurde:

$$(1)\quad \begin{matrix}\text{Calciumphosphat}\\(\text{Hydroxylfluorapatit})\end{matrix} + \begin{matrix}\text{Aluminium-}\\\text{Eisen(3)-}\end{matrix}\text{Hydroxyd} \rightleftharpoons$$

$$\rightleftharpoons \begin{matrix}\text{Aluminium-}\\\text{Eisen(3)-}\end{matrix}\text{Hydroxydphosphat} + \text{Calciumhydroxyd}.$$

Weiterhin wurde gezeigt, dass bei alkalischer Reaktion das Gleichgewicht nach links, bei saurer Reaktion nach rechts verschoben wird. Calciumgebundene Phosphorsäure wird also in aluminium- und eisen(3)-gebundene Phosphorsäure übergeführt und umgekehrt.

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* s. Fußnote S. 161

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DYNAMIK DER STICKSTOFFMINERALISATION IM ORGANISCHEN WALDBODENMATERIAL

I. BEZIEHUNG ZWISCHEN BRUTTOMINERALISATION UND NETTOMINERALISATION

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EINLEITUNG

Im Wald steht von den Hauptnährelementen Stickstoff häufig nur unzureichend zur Verfügung und begrenzt daher maßgeblich das Wachstum [43 44 22 40 34 49]. Zur Verbesserung der Stickstoffversorgung können in der Forstwirtschaft neben Anwendung von Stickstoffhandelsdüngern eine Reihe weiterer Maßnahmen dienen, die die Mineralisation des im Waldboden organisch gebundenen Stickstoffs erhöhen. Hierzu ist eine genaue Kenntnis der Faktoren nötig, von denen die Mineralstickstoff-Nachlieferung abhängt. Wir versuchten deshalb durch zahlreiche Brutversuche [9 42 18 35] Einblick in die Dynamik der Stickstoffmineralisation verschiedener Waldhumusformen zu gewinnen.

Die am Mineralisationsgeschehen beteiligten Mikroorganismen sind in ihren Leistungen von zwei Faktorengruppen abhängig: den „äusseren Faktoren", d.h. hauptsächlich Witterungseinflüssen und den „inneren Faktoren", d.h. im wesentlichen edaphischen Bedingungen. Die „äusseren Faktoren", wie Temperatur, Feuchtigkeit, Belüftung u.a.m. verändert man bei der Probenahme zum Brutversuch. Ihre Wirkung auf die N-Mineralisation ist bereits früher in Arbeiten über die Brutversuchsmethodik geschildert [47 48]. Den Einfluss von „inneren Faktoren", unter denen die chemische Zusammensetzung des organischen Materials und seiner mineralischen Beimengungen eine grosse Rolle spielt, untersuchen wir nachfolgend in Modellversuchen.

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Bei Arbeiten über die Dynamik der N-Mineralisation ist es nötig, sowohl die Nettomineralisation als auch den Bruttoumsatz [11 12 45 47] zu erfassen. Die im Brutversuch angehäuften N_{min}-Mengen * stellen ja keineswegs den gesamten mineralisierten Stickstoff dar [14 36 11 12]. Mit dem *Ab*bau organischen Materials ist stets ein *Auf*bau von Mikroorganismenmasse verknüpft. In ihr wird ein Teil des mineralisierten Stickstoffs sofort wieder festgelegt (Immobilisation). In Anlehnung an Jansson [11] können wir sagen, dass die im Brutversuch feststellbare N_{min}-Nettomenge nur ein Teil der Gesamtmineralisation, der sog. Bruttomineralisation ist.

Neben dieser mikrobiellen N-Immobilisation kann im Waldhumus unter geeigneten Bedingungen auch N_{min} in neu sich bildende Huminsäuren eingebaut werden [14 10 31 43 24 37]. Hierdurch ist ebenfalls eine Verminderung der Nettoanhäufung bei hohem Bruttoumsatz gegeben. Ausserdem ist zu berücksichtigen, dass Ammoniumionen im Boden durch Fixierung im Gitter von Tonmineralen festgelegt werden [1].

Durch die Änderung eines Faktors, der die N-Mineralisation beeinflusst, ist eine Verschiebung im Verhältnis Bruttomineralisation: Nettomineralisation möglich. Vollzieht man solche Änderungen unter definierten Bedingungen, so ist mit Einblicken in den genannten Zusammenhang zu rechnen. In unseren Modellversuchen prüften wir zunächst den Einfluss einer Zugabe von leicht angreifbaren, aber N-freien organischen Substanzen. Weiterhin untersuchten wir Proben, in denen als Folge von Düngungsmaßnahmen ein gesteigerter Bruttoumsatz zu erwarten war. Da auch bei einer Besserstellung der „äusseren Faktoren", wie sie bei jedem Ansetzen der Proben zur Bebrütung geschieht, eine Veränderung des Verhältnisses von Brutto- zu Nettomineralisation anzunehmen ist, bezogen wir diesen Vorgang ebenfalls in die hier folgenden Betrachtungen mit ein. Endlich erhöhten wir durch mechanische Zerkleinerung der Brutproben die mikrobiell verfügbaren C-Quellen und analysierten die Wirkung dieser Maßnahme.

METHODIK UND VERSUCHSMATERIAL

Wir entnehmen die Proben für den Brutversuch durch Ausstechen zahlreicher Einzelproben, die wir zu einer Mischprobe vereinigen. Nach möglichst kurzer Transportdauer sieben wir das noch naturfeuchte Material. Es wird

* N_{min} = Mineralstickstoff (NH_4-N + NO_3-N).

in gleicher Schichthöhe in ¼ Ltr.-Geleegläser gefüllt und auf 60% der maximalen Wasserkapazität [41] gebracht. Die Bebrütung erfolgt bei 20° Celsius in einem dunklen Feuchtraum. Der Wassergehalt der Proben wird nötigenfalls laufend ergänzt. Weitere methodische Einzelheiten schilderten wir an anderer Stelle [47 48]. Hält man die genannten Bedingungen konstant, so liefert der Laborbrutversuch reproduzierbare Werte [48].

Die Analysen auf den im bebrüteten Material angehäuften N_{min} führen wir jeweils in 1- bis 3-wöchigem Abstand durch, um Zeitkurven zu erhalten. Wir verwenden immer den gesamten Inhalt eines Probeglases. Die feuchten Proben schütteln wir mit 1% iger $KAl(SO_4)_2$-Lösung aus. Im Filtrat bestimmen wir NH_4-N nach der Conway-Methode [6 4] durch Mikrodiffusion und nachfolgende Neßlerisierung photometrisch. NO_3-N messen wir ebenfalls photometrisch nach Anwendung der Xylenol-Methode [30] mit Wasserdampfdestillation. Gesamt-Kohlenstoff ermitteln wir reduktiometrisch [28], Gesamt-Stickstoff nach der Kjeldahl-Methode im Halbmikroverfahren. Alle angegebenen pH-Werte sind in 1*N* KCl-Lösung bestimmt (Röhrenvoltmeter mit Glaselektrode).

Die N-Bruttomineralisation ist ohne N^{15}-Technik nicht direkt zu messen. Da diese Untersuchungsmethode bei unserer Arbeit nicht angewandt werden konnte, zogen wir die CO_2-Produktion der Proben als Maßstab für die Bruttomineralisation heran. Zahlreiche Arbeiten [38 16 17 25] haben erkennen lassen, dass die gebildete CO_2-Menge einen brauchbaren Maßstab für die gesamte Mikroorganismentätigkeit darstellt. Von ihr ist aber im wesentlichen die Höhe des Bruttoumsatzes der Mineralisationsvorgänge abhängig. Jansson [12] konnte diesen Zusammenhang in seinen Versuchen mit N^{15}-Technik klar nachweisen.

Wir stellen die Höhe der CO_2-Produktion der Brutproben in meist wöchentlichem Abstand durch kurzfristige Messungen fest. Dabei benutzen wir das Gasanalysengerät „Ultra-Gas" mit Kompensationslinienschreiber der Fa. Wösthoff [15]. Der zur automatischen Messung eingerichtete Apparat ist mit einem Meßstellenumschalter für 6 Anschlüsse ausgestattet. Während der CO_2-Messung verschliessen wir die Probegläser mit doppelt durchbohrten Stopfen. Eine Öffnung verbinden wir mit einer Meßstelle des Apparates, während durch die andere beim Absaugen durch das Gerät CO_2-freie Luft nachströmen kann. Abb. 1 zeigt die Messanordnung. Bei der von uns gewählten Messweise sammelt sich jeweils 25 Minuten lang CO_2 im geschlossenen Probegefäss an und wird dann 5 Minuten zur Analyse abgesaugt. Wir erhalten auf diese Weise Momentwerte der CO_2-Konzentration. Die gemessene CO_2-Konzentration ist in den Darstellungen der Analysenergebnisse stets auf die in den Probegefässen enthaltene Menge C bezogen, da nur so ein Vergleich von Bodenproben mit verschiedenem C-Gehalt möglich ist. Die gewonnenen Messdaten sind CO_2-*Konzentrations*werte. Verbindet man sie zu einer Zeitkurve, so stellt die von der Kurve umfasste Fläche einen Maßstab für die produzierte CO_2-*Menge* dar. Die bei der Messung abgesaugte Luftmenge ist bekannt. Sie bleibt ebenso wie die übrigen Versuchsbedingungen stets gleich. Daher ist eine Berechnung der ausgeschiedenen CO_2-Menge in absoluten Zahlen möglich. Sie geschieht mit einem experimentell ermittelten

Umrechnungsfaktor. Da es Momentwerte sind, streuen die CO_2-Zahlen stärker als die über mehrere Wochen summierten Werte der N_{min}-Anhäufung. Um dies auszugleichen, führen wir jeweils fünf Parallelbestimmungen durch.

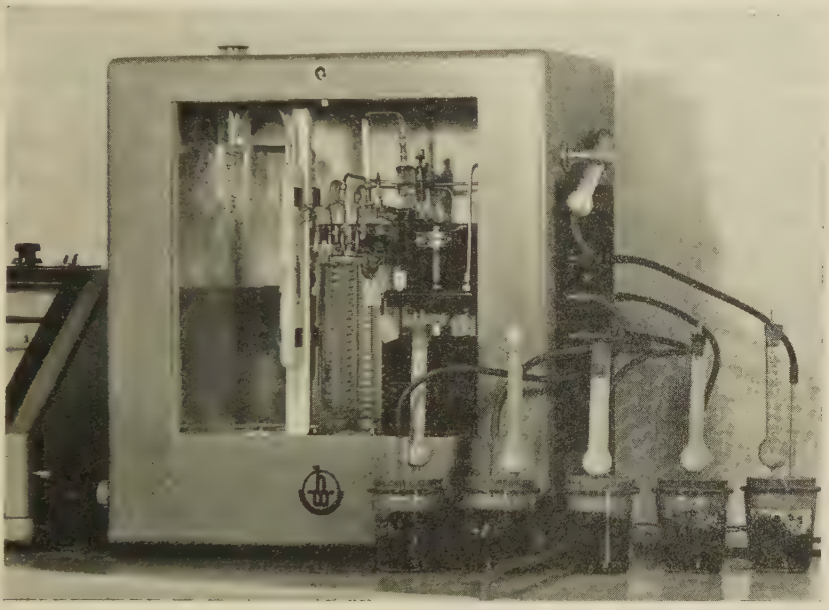

Abb. 1. Messanordnung zur Bestimmung der CO_2-Produktion. Rechts im Bild die während der Messung durch Gummistopfen mit Natronkalkröhrchen verschlossenen Brutgläser, in der Mitte das Messgerät, ganz links der Kompensationslinienschreiber. Einer der 6 Messanschlüsse des Geräts dient zur 0-Punkt-Ermittlung und saugt durch ein Natronkalkröhrchen an. Weitere Beschreibung im Text.

Das Versuchsmaterial wurde in Bayern und zwar überwiegend im Bereich von staatlichen Forstämtern entnommen (vergl. [49]). Es stammt von sehr frischen bis trockenen * Waldstandorten, deren Bodentyp, Grundgestein und Bestockung sehr verschieden sind. Staunasse oder grundwasserbeeinflusste Standorte blieben unberücksichtigt. Die Proben bestehen bei organischer Auflage (Rohhumus, Moder) meist aus der F-Lage und dem unteren Teil der L-Lage **. Bei Mull und mullartigen Humusformen umfassen sie den obersten, humusreichen Teil des A_1-Horizontes. In manchen Fällen sind auch die einzelnen Humuslagen getrennt entnommen. Wo nötig, ist das Versuchsmaterial in den Unterschriften zu den Figuren noch näher charakterisiert.

* Einstufung des Wasserhaushaltes nach Krauss [19].

** Die Bezeichnung der Humusform und der Humuslagen erfolgt nach Kubiena [20] (S. 27 u. 37), und Laatsch [21] (S. 116).

ERGEBNISSE UND DISKUSSION

1 *Direkte Erhöhung des mikrobiell leicht verfügbaren, stickstoffreien organischen Anteils durch Zugabe von Saccharose*

In diesen Modellversuchen wählten wir wasserlösliche Saccharose als mikrobiell leicht verfügbare und N-freie organische Substanz. Wir setzten sie zu Proben verschiedener Humusform zu. Fig. 1 zeigt als ein Beispiel den Einfluss dieser plötzlichen Erhöhung der C-Quelle auf die N_{min}-Anhäufung und CO_2-Produktion * einer Rohhumusprobe.

In den Proben steigt die CO_2-Entwicklung schon in den ersten

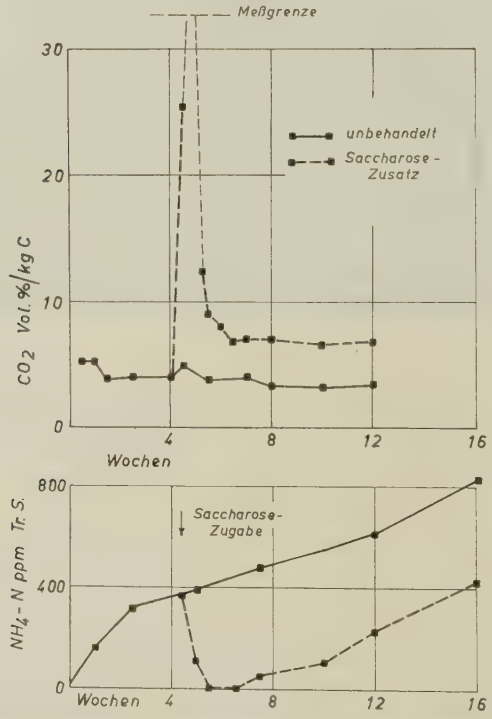

Fig. 1. Änderung der N_{min}-Anhäufung (nur NH_4-N-Bildung) und CO_2-Produktion von moderartigem Rohhumus „Sachsenried" (pH = 2,66; C : N = 27,2) nach Saccharose-Zugabe. (Die oberhalb der technisch bedingten Messgrenze liegenden CO_2-Werte sind nur geschätzt; die Kurve ist in diesem Bereich deshalb nur schwach gezogen.)

* Die CO_2-Werte in Fig. 1 sind ohne Berücksichtigung des zugesetzten Saccharose-C auf die in den Humusproben enthaltenen C-Mengen bezogen.

Stunden nach dem Einmischen der Saccharose jäh an. Nach dem Verbrauch des Zuckers sinkt die CO_2-Produktion wieder ab, läuft aber auf höherem Niveau als bei der unbehandelten Vergleichsprobe weiter. Die sicher stark vermehrten und lebhaft tätigen Mikroorganismen bewirken also auch in der Folgezeit einen erhöhten C-Umsatz (positiver „priming effect" [5 2 8 12]).

Der bis zum Zeitpunkt der Saccharose-Zugabe angehäufte Mineralstickstoff wird offenbar durch die schlagartig sich vermehrenden Mikroorganismen rasch aufgezehrt. Nach dem Verbrauch des zusätzlichen verfügbaren Saccharose-C beginnt die N_{min}-Anhäufung erneut in ähnlicher Weise wie vor dem Zuckerzusatz.

Erfolgt die Saccharose-Zugabe bereits bei Beginn des Brutversuchs, so spielen sich – wie weitere Modellversuche zeigten – grundsätzlich die gleichen Vorgänge ab. Mineralstickstoff gelangt erst zur Anhäufung, wenn die zusätzlich gebotene C-Quelle erschöpft ist. Bis zu diesem Zeitpunkt herrscht starke N-Festlegung, also hohe Bruttomineralisation bei völlig unterdrückter Nettoanhäufung. *Dieser Zustand tritt immer ein, wenn man mikrobiell verwertbares und N-armes organisches Material in grossem Überschuss anbietet. Der gesamte mineralisierte Stickstoff wird dann sogleich wieder in der sich vermehrenden Mikrobenmasse festgelegt* [39 13 29 3 26 32 33 27]. Da die höheren Pflanzen auf die Nettomineralisation angewiesen sind (vergl. [49]), spricht man in derartigen Fällen von „biologischer N-Sperre" [23 21]. Im Ackerboden kann man durch Einpflügen grösserer Strohmengen den gleichen Effekt hervorrufen.

2 *Indirekte Erhöhung des leicht verfügbaren, organischen Anteils durch Verbesserung der mikrobiellen Abbaubedingungen*

21 Untersuchung von am Standort gedüngtem, organischem Bodenmaterial.

Ebenso wie die Zugabe von leicht verfügbarer, organischer Substanz kann auch eine Mineraldüngung einschneidende Veränderungen im Verhältnis N-Bruttomineralisation zu N_{min}-Anhäufung bewirken. Ein Beispiel soll dies erläutern. Auf einer Versuchsfläche im Bayer. Forstamt Schwabach wurden auf einem armen Rohhumusstandort mit schlechtwüchsiger Kiefer Düngungsmassnahmen durchgeführt (Einzelheiten siehe Zöttl [46]). Ein Jahr später entnahmen wir Proben aus den L- und F-Lagen der unbehandelten Vergleichsparzelle und der mit kohlensaurem Kalk,

Hyperphosphat, Patentkali und Ammonsulfatsalpeter gedüngten Parzelle.

Die Proben der gedüngten Parzelle enthielten noch $CaCO_3$ in Form von Krusten und Bröckchen. Patentkali und Ammonsulfatsalpeter waren aus diesen oberen Humuslagen längst ausgewaschen oder von Wurzeln und Mikroorganismen aufgenommen. Ausser einer gewissen Schwärzung des Humus konnte man keine wesentlichen Veränderungen in der Materialzusammensetzung feststellen. Die Trockenheit des Standorts hatte bis zur Probenahme eine rasche Umsetzung des Kalks verhindert.

Beim Ansetzen zum Brutversuch wurde das organische Bodenmaterial innig gemischt und der kohlensaure Kalk dadurch erst homogen verteilt. Während des Brutversuchs (Fig. 2) läuft in der

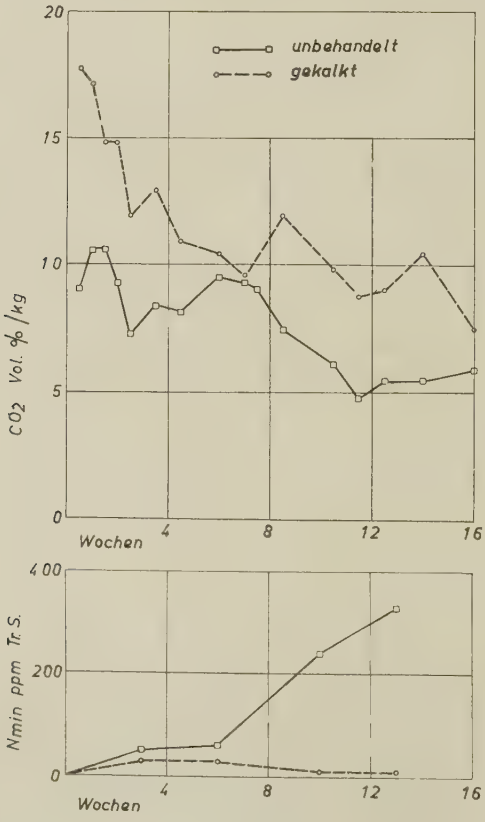

Fig. 2. N_{min}-Anhäufung und CO_2-Produktion in gekalkten (pH = 6,10) und unbehandelten (pH = 2,35) Brutproben von der Versuchsfläche „Schwabach" (nur Bildung von NH_4-N, keine Nitrifikation).

unbehandelten Probe nach einiger Zeit eine stetige N_{min}-Anhäufung an, während die gekalkte Probe auch nach fast 14 Wochen Versuchsdauer nur äusserst wenig Mineralstickstoff enthält. Ein Vergleich der CO_2-Kurven macht deutlich, dass in der gekalkten Probe eine höhere Bruttomineralisation herrscht. Es kommt zu keiner stärkeren Anhäufung von N_{min}, weil dieser zum grössten Teil sogleich wieder immobilisiert wird. Neben mikrobieller Festlegung ist bei diesem hohen pH-Wert auch Einbau von N in Humussubstanzen möglich.

Bei Proben mit hohem pH-Wert und Karbonatgehalt wären auch N-Verluste durch NH_3-Abdestillation denkbar. Dieser Vorgang könnte ebenfalls die in den gedüngten Proben beobachtete äusserst geringe N_{min}-Anhäufung verursachen. Wir prüften diese Möglichkeit von NH_3-Verlusten in geschlossenen Versuchsanordnungen, in denen etwa abdestilliertes Ammoniak festgestellt werden konnte. In keinem dieser Versuche war jedoch eine derartige Erscheinung nachzuweisen. Nach Gerretsen u. de Hoop [7] könnten unter ähnlichen Bedingungen, wie sie in diesem gedüngten Rohhumus herrschen, u.U. auch gasförmige N-Verluste durch Reaktion von Nitrit mit Ammoniumionen auftreten. Eine Anhäufung von Nitrit war in unserem Material aber nicht nachweisbar. Dieser Verlustquelle kommt daher wahrscheinlich keine Bedeutung zu.

Die gemessene geringe N_{min}-Anhäufung ist also wohl in erster Linie eine Folge starker biologischer N-Immobilisation, wie sie bei hoher Bruttomineralisation eintreten kann. *Eine solche Verschiebung in der Mineralisationsdynamik können wir somit durch verschiedene Maßnahmen hervorrufen: Direkt durch Zusatz von leicht verwertbarem C-Material oder indirekt durch Verbesserung der Lebensbedingungen der Mikroorganismen infolge des durch Düngung erhöhten pH-Werts. Im Extremfall wird dabei die N_{min}-Anhäufung fast ganz unterdrückt.*

22 Anlaufperiode der Mineralstickstoff-Anhäufung bei Beginn der Bebrütung.

Auch bei Verbesserung der „äusseren Faktoren" ist eine Verschiebung im Verhältnis Brutto- zu Nettomineralisation möglich. Wie an anderer Stelle [48] eingehend geschildert, zeigen Rohhumusproben in der ersten Zeit der Bebrütung oft nur eine geringe N_{min}-Anhäufung, die erst später stärker zunimmt (Kurventyp A in [48]). Wir wollen dieses Verhalten an einem besonders extremen Beispiel untersuchen.

In einem Brutversuch mit Rohhumus „Kellerhügel" (Fig. 3) setzte erst nach mehr als 6 Wochen Versuchsdauer eine geringe N_{min}-Anhäufung ein. Zugabe von Saccharose blieb, wie Fig. 3 zeigt, auf N_{min}-Anhäufung und CO_2-Produktion ohne Wirkung. Zugesetzter NH_4-N nahm im Laufe der Versuchszeit deutlich ab, wobei sich die CO_2-Entwicklung gegenüber der Vergleichsprobe anhaltend erhöhte. Bei der Serie mit N_{min}- *und* Saccharose-Zugabe zeigten sich der einsetzende N_{min}-Verbrauch und die Atmungserhöhung am deutlichsten.

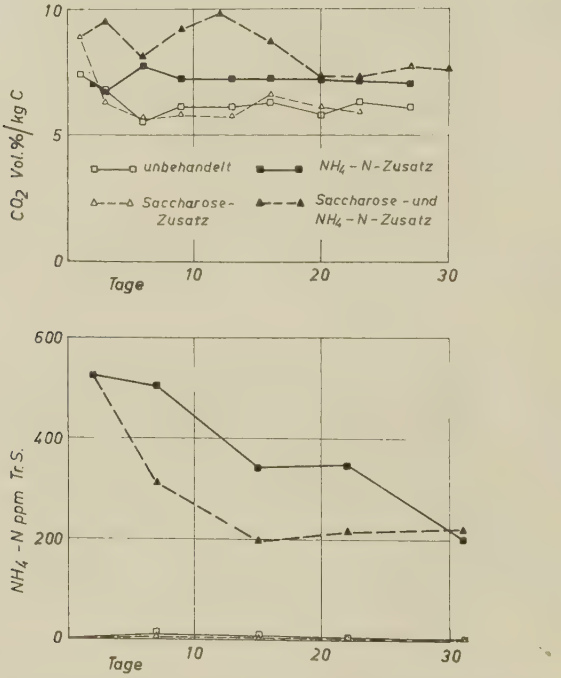

Fig. 3. N_{min}-Anhäufung und CO_2-Produktion von Rohhumus „Kellerhügel" (pH = 2,73; C : N = 44,2). 4 Versuchsglieder:

a) Unbehandelte Vergleichsprobe;
b) Zugabe von 200 mg Saccharose;
c) Zugabe von ca. 550 ppm NH_4-N;
d) Gemeinsame Zugabe von Saccharose und NH_4-N.

Aus diesen Resultaten geht hervor, dass die Anlaufperiode der N_{min}-Anhäufung im Brutversuch durch hohe N-Immobilisation bedingt ist. Zusatz von weiterem leicht angreifbarem C-Material

(Saccharose) bleibt ohne Wirkung, da in diesem Stadium noch relativ viel C-Verbindungen der Probensubstanz selbst mikrobiell verfügbar sind. Begrenzt wird der Abbau der organischen Substanz hier durch N-Mangel. Eine Zugabe von Mineralstickstoff (NH_4-N) führt deshalb zu erhöhter CO_2-Produktion, während der zugesetzte N_{min} selbst allmählich verbraucht wird. Gibt man gleichzeitig mit Mineralstickstoff noch Saccharose, so steigt die N-Immobilisation entsprechend an.

3 *Erhöhung des Anteils mikrobiell leicht angreifbaren organischen Materials durch mechanische Zerkleinerung.*

Im Brutversuch ist die zerkleinernde und durchmischende Wirkung der Bodentiere weitgehend ausgeschaltet. Durch das Sieben der Proben werden grössere Vertreter der Bodenkleinfauna, wie Lumbriziden, viele Insektenlarven, u.a. eliminiert. Auch ein Teil der mittelgrossen Gruppen passiert das Sieb nicht oder wird beim Durchreiben zerdrückt. Soweit Kleintiere mit in die angesetzten Proben gelangen, scheinen sie sich nach unseren Beobachtungen während der Bebrütungsdauer nie in bemerkenswertem Umfang zu vermehren. Ihre Beteiligung am Abbau der organischen Substanz dürfte im Brutversuch daher relativ gering sein.

Da eine weitere Aufbereitung durch die Bodenfauna also weitgehend fehlt, erschöpft sich die nach dem Sieben vor allem im Rohhumus zunächst relativ grosse Menge von mikrobiell leicht angreifbarem organischem Material doch bald weitgehend; die CO_2-Produktion fällt nach langer Brutdauer auf asymptotisch weiter sinkende geringe Werte ab. Wir versuchten nun den mikrobiell verfügbaren Anteil durch Mahlen von gesiebtem organischem Bodenmaterial zu erhöhen und prüften den Einfluss dieser Maßnahme auf das Mineralisationsgeschehen.

Hierzu wurden naturfeuchte, 5-mm gesiebte Proben aus der organischen Auflage von Waldböden in einer Condux-Mühle mittelstark gemahlen. Der Zerkleinerungsgrad der gemahlenen Proben ist aus Tabelle 1 ersichtlich.

TABELLE 1

Anteil der Siebfraktionen im gemahlenen und nicht gemahlenen Rohhumus „Amberg" (Angabe in Gewichts-% der Gesamtmenge. Die Trennung in die aufgeführten Siebfraktionen erfolgte am getrockneten Material)

Siebfraktion mm	>1	1–0,5	0,5–0,2	<0,2
Gemahlen %	3	9	17	71
Nicht gemahlen %	13	12	34	41

Trotz Wasserkühlung der Mahlscheiben erwärmte sich das Bodenmaterial etwas beim Mahlen. Dies dürfte der Grund für die bei Versuchsbeginn teilweise gefundenen NH_4-N-Mengen im gemahlenen Humus sein (Fig. 5). Das Mahlen veränderte das Aussehen der Proben beträchtlich. Auch grobfilziger, trocken-faseriger Rohhumus erschien nun schwärzlich und wirkte, obgleich man die gröberen Teilchen noch unterscheiden konnte, wie Mull.

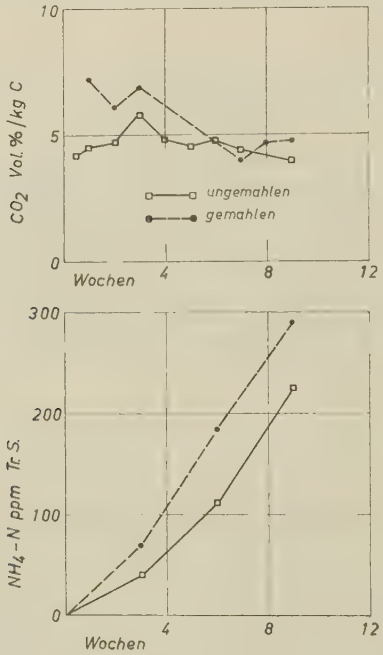

Fig. 4. N_{min}-Anhäufung (nur NH_4-N-Bildung) und CO_2-Produktion von ungemahlenem und gemahlenem Rohhumus „Amberg" (F-Lage; pH = 2,76; C : N = 30,5).

Ein Beispiel für die Versuche mit gemahlenen und ungemahlenen Proben aus der F-Lage von Rohhumusstandorten ist in Fig. 4 (Kiefern-Rohhumus „Amberg") dargestellt. Es ergab sich eine höhere CO_2-Produktion und N_{min}-Anhäufung in den gemahlenen Proben. Die Zerkleinerung hatte also den mikrobiellen Angriff auf die N-haltigen organischen Substanzen erleichtert und zu höherem Bruttoumsatz bei gleichzeitig verstärkter Nettoanhäufung von Mineralstickstoff geführt.

Beim Mahlen von Streulagen-Material (L-Lage) zeigte sich ein

anderes Ergebnis. Die zerkleinerten Proben aus der Streulage „Parsberg" (Fig. 5) hatten zwar zu Beginn der Bebrütung eine gegenüber der Vergleichsprobe höhere CO_2-Produktion. Sie sinkt dann aber rasch unter die Werte der ungemahlenen Probe ab (Fig. 5). Im gemahlenen Material wird die zunächst angelieferte NH_4-N-Menge rasch immobilisiert; im weiteren Verlauf der Bebrütung treten nur noch Spuren von Ammonium auf. Die CO_2-Entwicklung liegt auch beim ungemahlenen L-Lagen-Material absolut hoch (vgl. Fig. 5 mit Fig. 4 sowie den übrigen CO_2-Kurven). Obwohl nur die schon mehrjährige Streu entnommen wurde, enthält die Probe offenbar einen weit grösseren Anteil mikrobiell verfügbarer organischer Substanz als die stärker abgebaute F-Lage. Trotzdem erhöht sich dieser Anteil, wie die anfänglich starke CO_2-

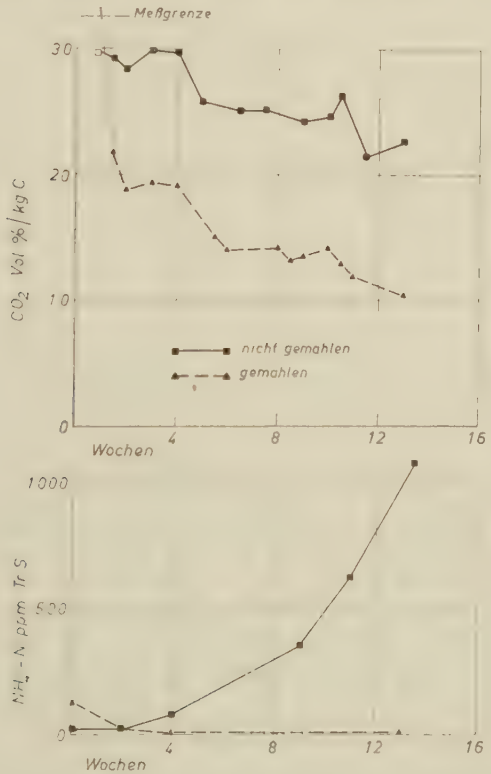

Fig. 5. N_{min}-Anhäufung und CO_2-Produktion von nicht gemahlenem und gemahlenem Streulagen-Material des Moders„ Parsberg" (pH = 3,65; C : N = 31,8).

Produktion der gemahlenen Probe zeigt, durch das Zerkleinern noch beträchtlich. Er unterliegt indessen einem sehr raschen Verbrauch, so dass die CO_2-Kurve bald unter die Vergleichswerte der ungemahlenen Probe sinkt. *Das Zerkleinern der noch relativ wenig abgebauten Streu hat also einen explosionsartigen Anstieg der Bruttomineralisation zur Folge, während gemahlenes F-Lagen-Material nur geringere Ausschläge zeigt.*

Die Menge mikrobiell verfügbarer organischer Substanz und ihr N-Gehalt bestimmt auch hier den Umfang der N_{min}-Anhäufung. Bei der gemahlenen Streulage „Parsberg" bleibt diese Anhäufung verschwindend gering, da im weiteren Bebrütungsverlauf der Mikroflora offenbar immer noch relativ viel N-freies organisches Material

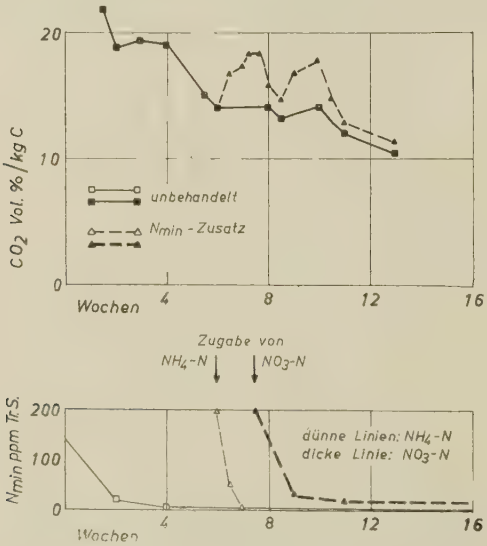

Fig. 6. N_{min}-Gehalt und CO_2-Produktion von gemahlener Streulage „Parsberg" nach Zusatz von NH_4-N und NO_3-N.

zur Verfügung steht. Dies lässt die mit vorübergehender Erhöhung der CO_2-Produktion verbundene rasche Immobilisation von zugesetztem NH_4-N und NO_3-N erkennen (Fig. 6). Die Mikroflora dieser gemahlenen Streulagen-Probe verwertet im Stadium der biologischen N-Sperre sogar den angebotenen Nitrat-Stickstoff, der sonst in nicht nitrifizierendem Material bei gleichzeitigem NH_4-N-Angebot nicht angegriffen wird [47] [50].

Wir können zusammenfassend feststellen, dass jede Verbesserung

der Lebensbedingungen der Mikroorganismen in den untersuchten Waldhumusproben grossen Einfluss auf die N-Mineralisation hat. Stellt man „innere" oder „äussere" Faktoren günstiger, so bewirkt dies mindestens zunächst immer eine Erhöhung der Bruttomineralisation bei gleichzeitiger Reduzierung der N_{min}-Anhäufung. Die Intensität der einsetzenden „biologischen N-Sperre" hängt einmal vom Ausmass der vorgenommenen Faktorenänderung ab. Zum anderen wird sie durch die Menge der im untersuchten Bodenmaterial vorhandenen N-freien Kohlenstoffverbindungen beeinflusst. Diese sind in Waldhumusformen mit stockender Zersetzung stets in grossem Umfang enthalten.

ZUSAMMENFASSUNG

In Brutversuchen mit verschiedenartigem Humusmaterial von Waldstandorten in Bayern wird die Beziehung zwischen Brutto- und Nettomineralisation des organisch gebundenen Stickstoffs untersucht.

Welcher Anteil der Bruttomineralisation als Nettobetrag im Brutversuch zur Anhäufung gelangt, hängt von der stofflichen Beschaffenheit des organischen Materials („innere Faktoren") und von den gesamten Lebensbedingungen der am Mineralisationsgeschehen beteiligten Mikroorganismen ab. Hat das Humusmaterial von Natur aus einen hohen Gehalt an mikrobiell angreifbaren stickstoffreien Substanzen, so erscheinen auch bei hoher Bruttomineralisation lediglich geringe Nettomengen. Gleiches gilt, wenn man den Prozentsatz stickstoffreien Kohlenstoffmaterials durch die Zugabe von verwertbaren Kohlenhydraten künstlich erhöht.

Eine Änderung der Lebensbedingungen der Mikroorganismen durch Düngung steigert die Stickstoff-Immobilisation bei hohem Bruttoumsatz. Man kann diesen Zustand einer im Extremfall verschwindend geringen Nettoanhäufung bei hoher Bruttomineralisation („biologische Stickstoffsperre") wenigstens vorübergehend auch durch eine Erhöhung der mikrobiellen Umsatzaktivität infolge günstiger „äußerer" Lebensbedingungen hervorrufen. Dies geschieht beim Ansetzen der Proben zum Brutversuch und kommt in der Anlaufperiode zum Ausdruck, während der sich nur sehr wenig Mineralstickstoff anhäuft.

Die mechanische Zerkleinerung von organischem Material hat insbesondere bei Streulagen eine starke Steigerung der Bruttomineralisation zur Folge. In welchem Maße sich dabei die Nettoanhäufung ändert, ist vom Stickstoffgehalt der durch die Zerkleinerung zum mikrobiellen Abbau freigegebenen Substanzen abhängig.

SUMMARY

The relation between gross- and net-mineralization of organic nitrogen was investigated in laboratory incubation studies with different humus samples from forest sites in Bavaria.

The portion of the gross-mineralization which is accumulated as net-amount in the incubation test, depends on the composition of the organic matter ("internal factors") and the general living conditions of the micro-organisms which participate in mineralization. If the humus naturally contains a high amount of non-nitrogenous material, which is available to micro-organisms only low net-amounts are shown even at high gross-mineralization .The same result is shown by increasing the percentage of nitrogen-free organic matter by adding available carbohydrates.

Changing the environment of the micro-organisms by fertilization increases the nitrogen immobilization during high gross-mineralization. In extreme cases this state of very low net-accumulation during high gross-mineralization ("biological nitrogen blockade") can be produced, at least for a short time, by increasing the microbial activity by improving the environment. This happens in starting the incubation of the samples and shows itself in the lag period of the mineral nitrogen accumulation.

The mechanical breaking-up of organic matter results in a marked increase of the gross-mineralization, especially with samples of the litter layer. The extent of change in the net-mineralization depends on the nitrogen content of the organic matter, which becomes available to the micro-organisms by its disintegration.

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